A Fluorescence *In-Situ* Hybridisation and Molecular Investigation of Leukaemia Associated Abnormalities of Chromosome 6q.

A thesis submitted for the degree of Doctor of Philosophy by

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Abstract

Deletions of the long arm of chromosome 6 are a recognised recurrent cytogenetic abnormality associated with ALL. Balanced rearrangements of 6q are less common but occur in leukaemias of both lymphoid and myeloid origin. With the aim of identifying genes that contribute to leukaemia through loss or rearrangement the breakpoints of translocations and deletions of 6q were mapped by FISH. Comparison between the mapped deletions led to the identification of a 4.8 Mb CDR and candidate tumour suppressor gene (*GluR-6*). Expression of *GluR-6* was demonstrated to occur in haematologic tissues and mutation analysis was performed on leukaemic samples with clonal deletions of the region. In one of 14 cases a base pair substitution that led to a change in amino acid sequence was found. The base pair substitution was also present in the patients' remission sample but was not seen in any of 20 other normal bone marrow samples analysed. Analysis of the translocations identified a variety of breakpoints between 6q15 and 6q27, with none positioned within the CDR. In seven cases breakpoints clustered within a 14 Mb region of 6q22-q23 but different sub-regions were defined for five analysed in detail. Complex rearrangements in one case of ALL appeared to result in translocation of one homologue of 6q and deletion of the second. Detailed FISH analysis defined a translocation breakpoint falling between two PACs that contained exons of a known tumour suppressor gene, the *IGF2-R*. Southern blot analysis failed to confirm disruption of the *IGF2-R,* but an *IGF2-R-MRP8* fusion transcript was cloned by RACE PCR from the patients' c-DNA. Involvement of MRP8 in the translocation remains in doubt because attempts to confirm the presence of the fusion transcript were unsuccessful.

Table of Contents

Table of Figures

Table of Tables

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Chapter 1 (Introduction)

Cytogenetic rearrangements of the long arm of chromosome 6 frequently accompany malignant transformation of a number of tissues including breast, liver, and the urinogenital, nervous, endocrine and haematopoietic systems. Four genes on 6q have been cloned from leukaemia-associated translocations, while other balanced rearrangements are as yet uncharacterised. Among malignancies of lymphoid origin, deletions of 6q are common and it is widely assumed that loss of one or more tumour suppressor genes underlie these abnormalities. The principle aim of this study is to identify genes on 6q that contribute to haematopoietic malignancy through loss or rearrangement.

Section **1.1** of this chapter provides a broad overview of the genetic changes that accompany malignancy, concentrating on common features rather than details of specific diseases. Next, normal blood cell development, with emphasis placed on the lymphoid and myeloid lineages, has been briefly described (**1.2**). The classification of leukaemia is dealt with in section **1.3** and associated cytogenetic changes are described in section **1.4**. Taking specific, well researched examples to illustrate important general principles, the molecular consequences of the cytogenetic rearrangements are also discussed. In section **1.5** the classical tumour suppressor gene model and related concepts are described. Cytogenetic and molecular investigations of 6q that form the background to this project are covered in **1.6** and **1.7**.

1.1 The Genetic Basis of Malignancy.

It is now widely accepted that progression from a normal cell to a malignant clone is accompanied by the accumulation of genetic changes. That alterations to DNA played a significant role in carcinogenesis was long suspected because exposure to mutagenic chemicals also resulted in tumour formation in animals and man. Further support for the hypothesis came from the demonstration that a single gene from the rous sarcoma virus (src) could transform cells *in vitro*[1]. Subsequently it was demonstrated that fragments of DNA

from a human cancer, transfected into mouse NIH-3T3 cells, also induced the phenotypic changes associated with malignancy. The recovered transforming DNA was found to encode a mutated form of the *HA-ras* gene[2]. In the same fashion, other genes were implicated in malignancy and in their mutated and non-mutated forms respectively were termed oncogenes and proto-oncogenes.

A second line of evidence supporting the involvement of specific genes in the development of malignancy came from the analysis of banded metaphase chromosomes from human cancers. The Philadelphia (Ph) translocation [t(9;22)(q34;q11)] was found consistently in bone marrow cells from chronic myeloid leukaemia patients[3,4]. Following this discovery, other translocations have been associated with a wide range of different malignancies[5]. Molecular analysis of the break points of translocations lead to the identification of new proto-oncogenes that were activated through two basic mechanisms. First, ectopic or up-regulated gene expression could result from the replacement of wild type with novel regulatory sequences. Secondly, fusion of coding sequence from two different genes resulted in production of chimeric proteins with transforming properties[6].

Oncogenic proteins resulting from mutation or chromosomal rearrangements can be thought of as possessing dominantly acting gain of function properties. Because a single change of this type transformed NIH 3T3 cells, it was at first thought that just one mutation could cause human cancer. However, non-transfected NIH 3T3 cells are now thought to harbor mutations that contribute to a pre-malignant state. Etiological studies and evidence from animal models suggest that at least 4 to 6 different mutations are required for the development of most cancers[7]. Full malignant transformation depends on the acquisition of an appropriate combination of gain of function but also loss of function genetic changes. Loss of function mutations affect tumour suppressor genes (tsgs) that in the wild type form encode proteins that constrain neoplastic expansion. Tsgs were first recognised when inherited inactivating mutations of the Rb gene were found to be unmasked by tumour-associated deletions of chromosome 13 in cases of familial retinoblastoma[8].

Although certain translocations or mutations are strongly associated with or even diagnostic for a specific disease, apparently identical malignancies often have very different profiles of underlying genetic abnormalities. The range of known cancer related abnormalities at the cytogenetic and DNA sequence level is now huge. Paradoxically with growing appreciation of this genetic heterogeneity has come the realization that the functional consequences of the mutations involve the acquisition of only a small number of changes in cellular physiology (reviwed by Hanahan D. and Weinberg R.A.[9]).

It has been suggested that malignant cell growth is associated with disruption to a relatively small number of regulatory circuits governing the acquisition of only the six following essential cellular characteristics: 1) self-sufficiency in growth signals; 2) insensitivity to growth inhibitory signals; 3) evasion of apoptosis; 4) limitless replicative potential; 5) sustained angiogenesis and 6) tissue invasion and metastasis.

Of the six characteristics listed above two, (sustained angiogenesis and tissue invasion and metastasis), are of less relevance to leukaemias than to solid tumours and will not be covered in this thesis. Although signaling pathways contributing to the remaining characteristics are often difficult to place within one of the categories alone, the above classification will be used as a framework for a general discussion of malignancy. An additional type of mutation, that increases the rate of acquisition of the six types of change, will also be considered (**1.1.5**).

1.1.1 Self-sufficiency in growth signals.

Normal cells require mitogenic growth signals for active proliferation to occur. The signals are transmitted into the cell by transmembrane receptors that are, either tyrosine kinases or coupled to G-proteins[10]. The signalling molecules may be diffusible growth factors, extracellular matrix components or cell-to-cell interaction molecules. Neoplastic cells circumvent dependence on exogenous growth signals in a number of ways. Some tumours secrete their own growth factors, others upregulate or switch the type of transmembrane receptors produced making them hyper-responsive to low levels of growth factor[11,12]. Structurally altered receptors that are Constitutively activated, have also been reported[13].

Independence from growth factor control can also result from disruption of the complex signalling pathways that lie downstream of ligand activated growth factor receptors. A central component of these regulatory pathways is the SOS-Ras-Raf-MAPK cascade. Approximately 25% of tumours express structurally altered Ras proteins that release mitogenic signals in the absence of stimulation by their upstream regulators[14]. A second important growth factor regulated pathway involves activation of phosphatidylinositol 3-kinase (PI3K). PI3K, is activated not only by growth factors but also by Ras, and plays an anti-apoptotic role (see **1.1.3**)[15].

Activation of the growth factor regulated signal transduction pathways ultimately leads to the nuclear translocation of mitogen activated protein kinases (MAPKs) that phosphorylate and activate transcription factors governing growth and differentiation. The target genes may be general factors required for transition through the cell cycle such as the cellular cyclins and cyclin dependent kinases (detailed in **1.1.2**) or may code for proteins that drive differentiation of a specific lineage. As it might be expected, autonomy from growth factor regulation can also result from mutations occurring at the transcription factor level. Translocations that generate oncogenes from lineage specific transcription factors, play a particularly prominent role among the acute leukaemias and will be discussed further in section **1.4**.

1.1.2 Insensitivity to growth inhibitory signals.

The important growth and apoptotic pathways discussed in this section and in **1.1.3,** are represented diagrammatically in **Fig 1.1**.

Normal cell proliferation is regulated not only by positively acting growth factors but also by multiple growth inhibitory signals. Antigrowth signals block proliferation by two distinct mechanisms. Firstly, cells may be forced into a quiescent (G_0) state from which they can reemerge and continue cycling upon stimulation by appropriate factors and secondly they can enter a state of terminal differentiation or senescence in which proliferative potential has been lost.

Pathways that allow normal cells to respond to anti-growth signals are centered on factors governing transition through the G1 phase of the cell cycle. Most studied antiproliferative pathways are funneled through the retinoblastoma protein (pRB) which when hypophosphorylated blocks proliferation by binding E2F transcription factors. The E2Fs regulate transcription of a variety of genes needed for G1 to S phase transition. When bound to the E2Fs, pRB blocks transcription of their target genes by recruiting a histone de-acetylase. On phosphorylation, pRB is released from the E2Fs which then become positive regulator of cell cycle transition[16,17].

Phosphorylation of pRB is positively regulated by complexes of cyclin-D with cyclin dependent kinases 4 and 6 (CDK 4/6), and cyclin-E with CDK2. The ability of the cyclin-CDK complexes to form and phosphorylate pRB is antagonized by a series of CDK inhibitors known as INKs. Two of the best known INKs are $p15^{INK4B}$ and $p16^{INK4A}$ both of which perform a tumour suppressor function in a number of tissues[18,19]. The cyclin E/CDK2 complex is inhibited by WAF1 (also known as p21) that is transcribed under the influence of p53, a well characterised tsg. p53 is also a critical regulator of genes involved in the induction of apoptosis (see **1.1.3**), and the factors that cause p53 to induce cell cycle arrest in favor of apoptosis are at present uncertain.

The *RB* gene itself was identified as a tsg in families that carried a point mutation of one allele and developed bilateral retinoblastomas in which loss of the second allele resulted from cytogenetically detectable deletions of chromosome 13 (see also **1.5**). Mutations of *RB* have been found in some but not all other types of sporadic tumours and mice heterozygously inactivated for *Rb* develop pituitary adenomas[20]. The restricted range of tumours associated with *RB* mutations may in part be explained by a degree of functional redundancy between pRB and two related proteins, p107 and p130[21]. Cancer related inactivating mutations have been reported to occur in both additional RB family proteins[22].

As mentioned in **1.1**, exposure to growth factors prompts cells to enter S phase of the cell cycle in part by upregulating transcription of cyclins and CDKs. A second type of extracellular effector negatively regulates cell growth. Best studied of the antiproliferative

ligands that lead to pRB dephosphorylation is the transforming growth factor β (TGFβ). TGFβ causes synthesis of the $p15^{INK4B}$ and p21 proteins that block formation of the cyclin/CDK complexes responsible for pRb phosphorylation. Loss of function mutations of many genes in the TGFβ signalling pathway, including the TGFβ receptor, lead to human malignancies[23,24].

The pathways that lead to terminal differentiation are more diverse, tissue-specific and presently less well studied. One important mechanism for avoiding differentiation involves upregulation of the proto-oncogene *c-myc* that disrupts the balance between MYC-MAX and MAD-MAX protein complexes that in turn regulate the expression of differentiation signals[25]. The importance of disruption to lineage specific differentiation programs in leukemia is discussed in **1.4**.

1.1.3 Evasion of Apoptosis.

Programmed cell death or apoptosis occurs in normal cells in response both to the balance between external death and survival signals and to intracellular factors which sense DNA damage, signal imbalance and other changes such as hypoxia. Cells bearing oncogenic mutations are routinely destroyed through apoptosis and the disruption of apoptotic pathways probably occurs in all malignancies. The majority, though not all, of known apoptotic signals are transduced by the important tsg p53.

Loss of or inactivating mutations of p53 occur in about 70% of human malignancies and the remainder probably all have defects in genes functionally up or downstream of p53 [26]. The high rate of cancer-associated mutation of p53 reflects its central role in eliminating the genetic damage that underlies malignant transformation. Either of two routes may be activated by p53 on sensing potentially oncogenic lesions to DNA. In the first, cell cycle arrest occurs (see **1.1.2**) while DNA repair pathways are activated (see **1.1.5**). In the second pathway, caspase activation and apoptosis are initiated.

Central to the p53 pathway is a core regulatory circuit consisting of p53 itself, the ubiquitinating enzyme Mdm2, $p14^{ARF}$ and the E2Fs[27,28]. Under normal circumstances

ubiquitination by Mdm2, maintains p53 at low levels by targeting it to the lysosomes for degradation. In turn, p53 exerts transcriptional control over the level of Mdm2. In a second feedback loop, the E2Fs activate *p14^{ARF}* transcription and p14^{ARF} facilitates proteolytic degradation of the E2Fs. The two feedback loops are connected because p14^{ARF} inhibits Mdm2 mediated ubiquitination of p53 and p53 is a negative regulator of p14^{ARF} expression. Activation of a range of upstream sensors of DNA damage and some other pro-apoptotic signaling molecules, leads to post-translational modifications of p53 and/or Mdm2 that block ubiquitination of p53 and target it to the nucleus. Other pro-apoptotic signals, such as those transduced by activated oncogenes, indirectly regulate p53 through effects on transcription or activity of p14^{ARF}.

The ultimate effectors of apoptosis are the caspases that selectively destroy subcellular structures, organelles and DNA. Many signals that cause apoptosis converge on the mitochondria inducing cytochrome C release and caspase 9 activation[29]. Both pro-apoptotic (Bax, Bak, Bid, Bim) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W) members of the Bcl-2 family of proteins, act by controlling cytochrome-C release. p53 upregulates Bax in response to DNA damage and other intracellular abnormalities. Cytochrome-C release can also occur independently of p53 activation upon binding of the FAS and TNFα receptors by their ligands. Death signals are transduced by caspase-8 after the activated TNF-R1 and FAS receptors complexed with FADD cleave pro-caspase 8. Caspase 8 itself, has been reported to be silenced in cases of childhood neuroblastoma[30].

Examples of extracellular ligands that induce antiapoptotic (survival) signals are IGF-1 and IGF-2, through the IGF-1 receptor, and IL-3 through the IL-3 receptor. Both these ligands and some intracellular signals via Ras stimulate the anti apoptotic PI3 kinase-AKT/PKB pathway. Recently AKT has been shown to both activate Mdm2 and to prevent nuclear localization of p21, linking it to both the core p53 regulatory circuit and to pRb mediated control of the cell cycle[31,32]. This pathway is also modified in many cancers and the multi-lineage tsg pTEN negatively regulates AKT survival signals[33].

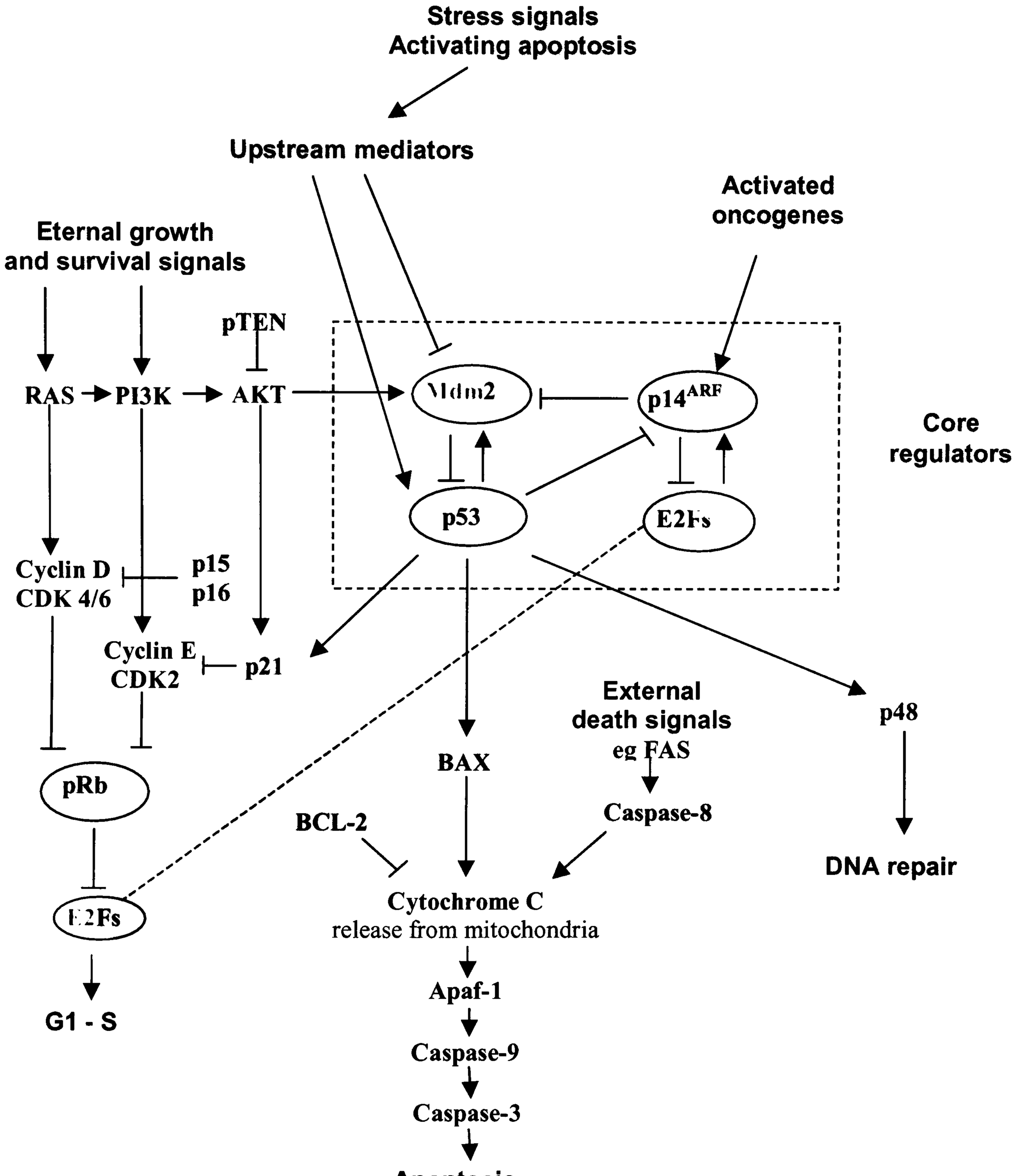


Fig 1.1 Major components of cellular growth and apoptotic pathways.
Oncogenes are shown in red and tumour suppressor genes in green.

It should be emphasized that only selected, relatively well understood, aspects of the control of growth and apoptosis are presented here. Although much of the detailed circuitry of the apoptotic pathways remains to be elucidated, it is becoming increasingly clear that a high degree of functional redundancy exists between both regulatory and effector components. One expected consequence of this redundancy is that successive mutations affecting the components of apoptotic pathways will have additive tumourigenic effects.

1.1.4 Limitless replicative potential.

All of the acquired characteristics so far described lead to evasion of limitations on growth placed by external factors. However, almost all normal cells are also intrinsically limited in their replicative potential. This limitation arises because with each successive round of replication 50-100 base pairs of DNA are lost from the ends (telomeres) of each chromosome. Each telomere is capped by several thousand hexanucleotide repeats that form a looped structure protecting the chromosome end from degradation and recombination. Telomeric repeats are completely eroded after about 60-70 rounds of replication leading to recombination between chromosome ends and the formation of genetically unstable dicentric chromosomes. The consequent disruption to the genome normally results in cell death. Therefore, in order to replicate beyond a finite cell number, malignant clones have to evolve mechanisms that restore telomere stability. In most tumours the enzyme telomerase, which adds hexanucleotide repeats to telomeres, is upregulated[34].

1.1.5 Loss of DNA maintenance mechanisms.

The pathways described in **1.1.1-1.1.4** include several tsgs that lie directly in the pathway of full malignant transformation and for this reason have been termed "gate keepers" [35]. Loss of function of a second group of genes, sometimes known as "caretakers", accelerates the rate of acquisition of mutations, including those that represent steps towards a cancer phenotype. The caretakers consist of genes involved in genome surveillance and the repair of DNA damager (reviewed by Hoeijmakers 2001 and Khanna and Jackson 2001[36,37]).

Several different classes of DNA damage are recognized and repaired by distinct sets of enzymes. Bulky DNA adducts and double strand breaks that cause major distortions to the double helix are sensed by general surveillance mechanisms. Smaller lesions are recognised because they stall transcription or replication. On sensing DNA damage two types of signals are transduced, those leading to the recruitment of repair apparatus to the site of damage and those preventing entry into S phase of the cell cycle. Much of the known repair signaling circuitry is centred on p53 (see **1.1.3**) which, depending on the nature of the detected damage and cell type involved, may trigger apoptosis rather than replication block.

Repair systems are of three main types, excision repair, double strand break repair (DSBR), and mismatch repair. In the first of these a damaged nucleotide, or patch of nucleotides surrounding the lesion, are excised by repair specific enzymes such as the Xeroderma Pigmentosa (XP) proteins. The resulting gap is then filled by the cells regular replication machinery using the complementary strand as a template[38,39].

DSBR is initiated when the ataxia telangiectasia mutated (ATM) protein detects a double strand break[40]. Two other proteins, ataxia telangiectasia related (ATR) and DNA protein kinase (DNA-PK), are then recruited to the broken end where they phosphorylate histones over a region of approximately one Mb[41]. If the break has occurred post-S phase the relatively error free process of homologous recombination, in which the sister chromatid is used as a template, takes place. A number of repair specific enzymes including NBS-1 BRCA1 and BRCA2 are involved in homologous recombination[42]. Double strand breaks arising prior to S phase, have no easily available template and are repaired through the error-prone process of end joining. Several proteins involved in antibody and T-cell receptor rearrangement (see **1.2**) are also used in the process of end joining. These include the KU70/80 end binding complex DNA-PK and XRCC4 DNA ligase[43]. End joining not only leads to the risk of recombination taking place between different chromosomes, particularly in cells that are actively rearranging antigens, but also to loss or gain of nucleotides.

Mismatch repair involves a number of proteins that recognize and rectify mismatch, insertion and deletion mutations introduced by DNA polymerase II. The process is tightly linked to

replication and involves protein complexes of the human homologues of *Drosophila* MutS and MutL (hMSH2/6 and hMLH/hPMS) that distinguish parent from daughter strand and recognize mutations. Excision of nucleotides in the daughter strand and re-synthesis of DNA is performed by specialized exonucleases and polymerases[44].

The important role of DNA repair systems in protecting against malignancy is highlighted by the existence of cancer prone syndromes in which germline mutation in a caretaker gene has occurred. Patients with Xeroderma Pigmentosum (XP) carry mutations affecting one of 7 different proteins (XPA-XPG) required for excision repair, are sun-sensitive and develop skin cancer[45]. Germ line mutations in the *ATM* gene lead to the condition of ataxia telangiectasia, an X-ray sensitive cancer-prone syndrome[40]. Other genes coding for proteins involved in DSBR that are known to be mutated in familial cancers include; *NBS-1* causing Nijimegen breakage syndrome, and *BRCA1* and *BRCA2* in breast cancer[42,46]. The *hMLH 1* and *2* and *hMSH6* genes, essential to mismatch repair, bear germ line mutations in more than half of hereditary non-polyposis colorectal cancer (HNPCC) patients. A range of sporadic tumours have also been associated with defects in mismatch repair[47].

Animal models also demonstrate that the DNA repair systems protect against the development of malignancy. $Ku80^{-/-}$ mice displayed a marked increase in chromosomal aberrations and slightly earlier onset of cancer. Double knock out $Ku80^{-/-}$ $p53^{-/-}$ mice developed lymphomas at an early age that, mirroring the situation in human lymphomas, were characterized by chromosomal rearrangements involving *IgH* and *c-Myc*[48]. Loss of Ku protein function also impaired the immune system and inactivation of DNA-Pk produces a similar but milder (skid) phenotype

1.2. Normal blood cell development.

Unless otherwise indicated the information in this section and section **1.3** is summarized from standard haematology texts[49].

Normal adult haemopoietic marrow is confined to the central skeleton and proximal ends of femurs and humeri. Stem cells committed to the myeloid and lymphoid lineages originate from a common (pluripotent) stem cell. After successive divisions a common myeloid progenitor gives rise to the erythroid, megakaryocytic, granulocyte/monocyte, eosinophilic and basophilic lineages. Two lineages, monocytes and neutrophils are derived from the granulocyte/monocyte line. The common lymphoid stem cell gives rise to T and B-lymphocytes. Pluripotent stem cells have the capacity for self-renewal and each can give rise to about 10^6 mature blood cells.

The bone marrow stroma is composed of a microvascular network and stromal cells which secrete a complex matrix of collagen and adhesive proteins. The adhesive proteins bind stem cells where they are presented with growth factors (often referred to as cytokines) produced by the stromal cells. Cytokines are also produced by T-lymphocytes, monocytes and macrophages and may circulate in the plasma or act through cell-to-cell adhesion. The direction in which stem and progenitor cells differentiate depends largely on the spectrum of growth factors to which they are exposed and the growth factor receptors they express. Cytokines may stimulate production of other growth factors or growth factor receptors. Other ligands play an inhibitory role, for example, TGFβ attenuates growth in a variety of haematopoietic cells. Although extracellular growth and inhibitory factors sustain haematopoiesis they are thought not usually to be instructive for lineage differentiation, but rather permissive for cell viability and proliferation (reviewed by Orkin 2000[50]). That is to say, in the absence of specific cytokines cells of a particular phenotype are blocked from entering the cell cycle or initiate apoptotic programs. As discussed in **1.2** and **1.3** the pathways that lie downstream of growth and inhibitory factor receptors have been linked to the core pRb and p53 pathways controlling cellular growth and survival. In the presence of appropriate permissive cytokines, cells bearing the right receptors are permitted to grow but differentiate under the control of intrinsic regulators of transcription. The transcription factors controlling haematopoietic differentiation have frequently been first identified through cloning break points of chromosomal translocations recurrent in leukaemia.

Transcription factors modulate expression of their target genes in a number of ways, some remodel DNA locally by ATP dependent mechanisms, others modify histones or interact with DNA polymerase II (reviewed by Lemon and Tjian 2000[51]). Frequently a number of proteins form multimeric complexes, that depending on the presence of co-activators or co-repressors can either be positive or negative regulators of transcription. Transcription factors are often classified according to their DNA binding domains. A group of helix-turn-helix containing transcription factors, known as the class I homeobox or HOX genes, play a critical role in embryonic development as well as in human haemopoiesis. The 39 HOX genes are organised into four groups A-D each one sharing common regulator sequences and occupying different chromosomal locations. Expression of genes within each cluster has a spatially/temporal relationship with the more 5' being expressed at early stages of development/haemopoiesis. The down stream targets of HOX genes remain largely speculative but it is clear that members of the HOX family regulate each other and related class II homeobox genes in a complex homeotic network (reviewed by van Oostveen and Bijl 99[52]). Regulatory sequences positioned upstream of *HOX* genes include TAAT and retinoic acid response elements. In *Drosophila* the *HOX* genes are regulated negatively by polycomb (Pc-G) and positively by trithorax (Trx-G) master transcription factors. In humans a single *Trx-G* homologue, the promiscuous proto-oncogene *mixed-lineage leukaemia* (*MLL*) has been cloned. Various *HOX* and also class II homeobox genes have themselves been shown to participate in chimeric fusion-oncogene formation or to be deregulated as the result of translocations in leukaemia.

In addition to *MLL* and the *HOX* family members, genes coding for several other transcription factors controlling blood cell development are recurrently interrupted or deregulated by leukaemia related chromosomal rearrangements. Proteins encoded by known oncogenes that also regulate transcription in the haematopoietic stem cell include TAL1, LMO2, HLXB9, GATA-2, CBFA2 and CBFβ. Development of the myeloid lineage is accompanied by expression of RARα, Myb and PU.1 and lymphoid restricted oncogene/ transcription factors include IKAROS, E2A and PAX 5 (for further discussion of the role of transcription factors in leukaemia see **1.4**).

1.2.1 Myeloid Development.

The leukaemias dealt with in this study originate from the granulocytic and lymphocytic lineages, so development of red blood cells and megakaryocytes are not discussed further. Under the influence of growth factors SCF, IL-3, GM-CSF, G-CSF, M-CSF and IL5 bone marrow stem cells are induced to differentiate into mature granulocytes (neutrophils, basophils and eosinophils) and monocytes.

Morphologically recognisable immature forms of the neutrophil lineage found in the bone marrow include promyelocytes, myelocytes, metamyelocytes and band neutrophils. Only mature neutrophils are found in normal blood and tissues where they perform a phagocytic function. Of the other granulocytic lineages eosinophil and basophil precursors are indistinguishable from those of the neutrophil lineage. Mature eosinophils perform specific roles in allergic response, defence against parasites and fibrin removal. Basophils mature into mast cells in the tissues. Immature forms of monocytes (promonocytes) can be recognised in the bone marrow. After migration to the tissues monocytes undergo further maturation into macrophages. Like neutrophils, monocytes and macrophages have a phagocytic function but also secrete growth factors (IL-1, TNF, IL-3, GM-CSF, G-CSF, M-CSF, IL-4 and IL-6) some of which may be targeted to tissues outside the haematopoietic system such as brain and liver. Specialised macrophages also act as antigen presenting cells to T-lymphocytes.

1.2.2 Lymphoid development.

Undifferentiated lymphoid cell expansion occurs in the bone marrow under the influence of cytokines: IL-1, IL-4, IL-6, TNF and IL-3. Growth of the B-cell lineage is under the control of IL-1, IL-4, IL-6, IL-7, IL-10, TNF, IL-2 and γIFN. T cells are stimulated to divide by IL-1, IL-2, IL-4, IL-6, IL-7 and IL-9 with later maturation occurring in the thymus. Both mature B and T cells express antigen recognising receptors on their cell surface, immunoglobulins (that are also the secreted antibodies) in the case B-cells and T cell receptors (TCRs) in the case of T cells. Antigen specificity results from rearrangement of genes that code for immunoglobulin and T cell receptor proteins during lymphocyte development. Immunoglobulins are composed of two heavy chains (IGH) encoded by a series of gene

segments on chromosome 14 band q32 and two light chains (IGL) either κ (chromosome band 2p11) or λ (chromosome band 22q21). In the germ line configuration the *IGH* and *IGL* consist of Variable (*V*) diversity (*D*), joining (*J*) and constant (*C*) regions each containing a number of different segments. During early B-cell development, under the influence of sequence specific recombinases, one of the *D* segments becomes joined with a *J* segment (in the *IGH*) and then to one of the *V* segments to form an actively transcribed *IGH* gene. Variations in the joining of *V*, *D* and *J* segments used and insertion of additional sequences by terminal deoxynucleotidyl transferase (TdT) at the *DJ* and *VD* junctions introduce additional diversity in the IGH repertoire. Similar rearrangements of *κ* and *λ IGL* genes take place slightly later in B-cell development. No *D* segments are present in the *IGL* genes.

T cell receptors are composed of heterodimers, most commonly composed of α and β chains or less frequently γ and δ chains. The genes encoding TCR α, β, γ and δ chains are composed of *V*, *D*, *J* and *C* regions and become rearranged during thymic T-cell development in a manner analogous to *IGH* and *IGL* rearrangement in B-cells.

Immature forms of B-cell present in the bone marrow include pre pro-B cells, pre-B cells, early B cells and mature B-cells. The most immature form of T cell found in the thymus is the Pre-T cell which gives rise to large cortical thymocytes, small cortical thymocytes and mature medullary thymocytes. Mature B and T cells leave the bone marrow and thymus and migrate to the lymphoid tissues where they are exposed to antigens by antigen presenting cells (APCs) in the lymphoid tissues, and at this point they are transformed into T or B immunoblasts. Some T immunoblasts go on to perform an immunocytotoxic function and then die. Others develop into memory T-cells capable of reacting more rapidly and intensely upon further exposure to antigens. B immunoblasts develop into lymphoplasmacytoid cells then immunoglobulin secreting plasma cells or alternatively develop into memory B cells.

Two forms of lymphocyte, natural killer (NK) cells and lymphokine activated killer (LAK) cells recognise and destroy foreign cells without producing antigen recognising receptors. NK cells recognise other structures on the target cell that in some cases are bound antibodies. LAK cells perform the same function but require activation by cytokines.

1.3 Classification of haematological malignancies.

The leukaemias can be broadly divided into those of myeloid and lymphoid origin. Diseases arising in the lymphatic system are categorised into Hodgkins and non-Hodgkins lymphomas. Chronic and acute forms of the leukaemias and lymphomas exist while the myelodysplastic syndromes (MDSs), polycythaemia rubra vera (PRV), essential thrombocytopaenia (ET) and myelofibrosis are usually classed as pre-leukaemias or smoldering leukaemias. The precise cell of origin of haematological malignancies is often uncertain and placing diseases into the above categories is not always straightforward. Rare cases of mixed-lineage leukaemia express immunological markers of both myeloid and lymphoid type and in leukaemia/lymphoma syndromes, disease of both the haematopoietic and lymphopoietic systems are manifest. Diagnosis of haematological malignancies is made on the basis of morphology, immunology and genotype as determined both by cytogenetic and molecular techniques.

1.3.1 Acute Myeloid leukemia (AML).

AML is usually classified according to the French, American, British (FAB) system into 7 morphologic sub-types (Table 1.1). The sub-types correspond to the lineage and stage of differentiation of the leukaemic clone. Several sub-types are associated with specific cytogenetic rearrangements (see **1.4.1**), further diagnostic and prognostic information can be derived from genotypic and immunological data.

1.3.2 Chronic myeloid leukemia (CML).

While the acute myeloid leukaemias generally result from maturation block of one or more of the myeloid lineages, CML involves the relatively indolent clonal expansion of mature or near mature granulocytes. If left untreated, the disease progresses to a more aggressive acute phase and finally blast crisis. Blast crisis of CML resembles acute leukemia of either lymphoid or myeloid lineage supporting the widely accepted belief that the disease arises in the pluripotent stem cell compartment. The *BCR-ABL* fusion gene resulting from the

Philadelphia (Ph) translocation [t(9;22)(q34;q11)] is considered diagnostic for CML and transformation to blast crisis is frequently accompanied by the acquisition of further cytogenetic abnormalities. Rare cases of *BCR-ABL* negative 'variant CML' have been described but are distinguished from CML by a somewhat different clinical course.

FAB Sub-Type	Lineage/ Stage of differentiation
M0	Undifferentiated common myeloid progenitor cells
M1	Myeloblasts without differentiation
M2	Myeloblasts with differentiation
M3	Promyelocytes
M4 and VM4	Myeloblasts and Monoblasts [variant(v) has eosinophilia]
M5	Monocytes
M6	Erythroblasts
M7	Megakaryoblasts

Table 1.1. French American British (FAB) classification of AML.

1.3.3 Myeloproliferative Disorders (MPDs) other than CML.

The MPDs, PRV, ET and myelofibrosis are considered non-leukaemic although they may occasionally transform into AML. All forms involve clonal expansion of the stem cells but in PRV the erythroid lineage is dominant, in ET there is excessive production of megakaryocytes and platelets while myelofibrosis results in a reactive fibrosis of the bone marrow. The three conditions are closely related and frequently one will transform into another.

1.3.4 Myelodysplastic syndromes (MDSs).

The MDSs comprise a spectrum of clonal disorders arising in the multipotent stem cell and leading to maturation defects and cytopaenias of all three myeloid lineages. There are 5 FAB sub-classes of MDS based upon numbers of blast cells in the bone marrow or peripheral blood, presence or absence of ring sideroblasts and the occurrence of monocytosis. The FAB sub-types are 1) Refractory anaemia (RA) 2) RA with ring sideroblasts (RARS) 3) RA with excess blasts (RAEB) 4) RA with excess of blasts in transformation (RAEB-t) and 5) Chronic myelomonocytic leukemia (CMML). The MDSs frequently transform into AML with an arbitrary level of 20% bone marrow blasts used to distinguish between the two diseases.

1.3.5 Acute Lymphocytic leukemia (ALL).

On the basis of morphology ALL can be classified into 3 FAB sub-types; L1: blasts are small and uniform with a high nuclear to cytoplasmic ratio. L2: large non-uniform blasts with a lower nuclear to cytoplasmic ratio irregular shaped nuclei and prominent nucleoli. L3: Large uniform vacuolated blasts with basophilia, regular nuclei and prominent nucleoli. The three sub-types show distinct age distributions with L1 accounting for 85% of childhood ALL and L3 only 1%.

ALL can also be classified according to lineage and stage of differentiation by using immunological markers. Immunological sub-types include common or early pre-B, pre-B, B and T ALL. The majority of childhood ALL are of the early pre-B immunological sub-type. ALL is associated with a number of characteristic numerical and structural cytogenetic abnormalities that provide the most important prognostic tool in this disease (see **1.4**). The stage of Immunoglobulin/T cell receptor rearrangement and segment usage has also been investigated in ALL and may provide additional prognostic information as well as a sensitive means of detecting relapse after treatment.

1.3.6 Chronic lymphocytic leukemia (CLL).

CLL is the commonest adult leukemia in western society. It is characterised by the accumulation of small mature lymphocytes in the blood, bone marrow and lymph nodes. Over 90% of CLL cases express surface immunoglobulin and 98% express B lineage markers the remaining 2% are of T cell origin. 50% of cases have cytogenetic abnormalities that together with presence or absence of specific immunological markers can aid in prognosis (see **1.4**)

1.3.7 Lymphomas.

The lymphomas result in malignant overgrowth of normal lymphoid tissue by abnormal cells that are usually of B or T lymphocyte origin, in later stages other tissues including the bone marrow may be infiltrated. The disease is usually divided into Hodgkin's disease (HD) and non-Hodgkin's (NHL) types. HD is characterised by the presence of large, multi-nucleated, Reed Sternberg cells that have been shown to be of B cell origin. Classification of NHL is complex and in a state of flux. The precise cell of origin is often unclear though most are rearranged either for the *IGH* and *IGL* or *TCR* genes. Since cases of lymphoma are not included in this study the classification of NHL will not be covered in detail in this thesis.

The lymphomas are characterised by complex cytogenetic rearrangements with translocations frequently resulting from illegitimate *IG* or *TCR* gene rearrangements. The presence of specific cytogenetic abnormalities, immunological markers and viral infection are all used to guide the clinical management of lymphomas. Recently, morphologically indistinguishable, diffuse large cell lymphomas were classed into prognostic sub-groups on the basis of expression microarray data[53]. The microarray approach to characterising malignancies seems likely to gain in importance as the relevant technology becomes more accessible.

1.3.8 Multiple Myeloma.

Multiple myeloma (MM) results from a monoclonal bone marrow proliferation of mature, dysmorphic plasma cells. At present it remains uncertain whether the plasma cells develop in the bone marrow or are post germinal centre and later infiltrate the marrow. TNF and IL-1 secreted by the multiple myeloma cells lead to osteolytic lesions. The neoplastic cells express clonally rearranged Ig proteins on their cell surface that are secreted in the plasma (paraprotein) and urine (Bence-Jones protein). Multiple myeloma is characterised cytogenetically by translocations of the *IGH* and *IGL* genes[54].

1.4 Cytogenetic Abnormalities of Haematological malignancies.

Conventional G-banded analysis of haematological malignancies has revealed many lineage-specific as well as non-specific cytogenetic abnormalities. If the same abnormality is identified repeatedly in a disease it is described as recurrent, while previously unreported changes are termed sporadic. Molecular cloning of genes affected by recurrent changes has resulted in the identification of many oncogenes and tsgs contributing greatly to the understanding of the biology of malignancy. As karyotypic data for the haematological malignancies accumulates, cytogenetics has come to play an increasingly important role in diagnosis, prognosis and monitoring of residual disease. Fluorescence *in-situ* hybridisation (FISH) and the related techniques of comparative genomic hybridisation (CGH) and multicolour FISH (M-FISH) or spectral karyotyping (SKY) have increased the accuracy and sensitivity of cytogenetic techniques (reviewed by Kearney 99[55]). For the purpose of this thesis recurrent cytogenetic abnormalities will be divided into myeloid specific, multilineage and lymphoid specific.

1.4.1 Recurrent cytogenetic abnormalities restricted to myeloid malignancies.

AML karyotypes are typically characterised by balanced translocations and the formation of chimeric oncogenes (reviewed by Hayashi 2000[56]). The MDSs and MPDs are by contrast, more often associated with monosomy or deletion of specific chromosomes. The most important recurrent chromosomal abnormalities with their associated underlying molecular lesions (where known) are presented in table **1.2**. The Philadelphia translocation and translocations of 11q23 will be considered in section **1.4.2** since they are common to both myeloid and lymphoid disease. The mechanisms by which the various chimeric oncogenes contribute to development of the common neoplastic characteristics, discussed in **1.1-1.5**, have been the subject of intensive research in recent years and are becoming increasingly well understood. A detailed review of published work exploring downstream effects of leukaemic translocations is beyond the scope of this thesis as is a description of the incidence and clinical implications of each rearrangement. However, some of the numerically most important and well understood, rearrangements will be discussed.

The translocations associated with AML, unlike many seen in lymphoid malignancies, result in the formation of chimeric fusion-proteins with oncogenic activity rather than deregulation of wild type genes. The t(3;3)/inv(3)(q21;q26) is an exception to this rule since the rearrangements cause the Ribophorin 1 promoter to drive ectopic expression of *EVI1*. One or both of the partners of most of the fusion genes is a transcription factor (or co-factor) involved in the regulation of normal myeloid development. The role of some of these transcription factors in normal haemopoiesis was described briefly in **1.2**, their contributions to leukaemogenesis will be discussed further in this section.

Cytogenetic Abnormality	Fusion Gene[references]	Disease sub-types
t(6;9)(p23;q34)	*DEK-CAN(NUP214)*[57]	AML-M1,M2,M4,M5
t(16;21)(p11;q22)	*TLS(FUS)-ERG*[58]	AML, CML-bc
t(8;21)(q22;q22)	*AML1-ETO*[59]	AML-M2
t(16;21)(q24;q22)	*AML1-MTG16*[60]	t-AML, MDS
t(3;21)(q26;q22)	*AML1-EVI1*[61]	AML, CML-bc
t(3;3)/inv(3)(q21;q26)	*EVI1-Ribophorin 1*[62]	AML-M7,
t(16;16)/inv(16)(p13q22)	*CBFβ-MYH11*[63]	AML-M4Eo
t(15;17)(q22;q12)	*PML-RARA*[64]	AML-M3
t(11;17)(q23;q12)	*PLZF-RARA*[65]	Atypical AML-M3
t(7;11)(p15;p15)	*NUP98-HOXA9*[66]	AML-M2, M4, CML, MDS
inv(11)(p15q22)	*NUP98-DDX10*[67]	t-AML, t-MDS
t(1;11)(q23;p15)	*NUP98-PMX1*[68]	AML-M2
t(2;11)(q31;p15)	*NUP98-HOXD13*[69]	t-MDS
t(11;20)(p15;q11)	*NUP98-TOP1*[70]	t-MDS
t(12;22)(p13;q13)	*TEL-NM1*[71]	AML-M4
t(12;15)(p13;q25)	*TEL-TRKC*[72]	AML-M2
t(1;12)(q25;p13)	*TEL-ARG*[73]	AML-M3
t(9;12)(q34;p13)	*TEL-ABL*[74]	AML-
t(7;12)(q36;p13)	*TEL-HLXB9*[75]	AML-M1, M3
t(8;16)(p11;p13)	*MOZ-CBP*[76]	AML-M4, M5
inv(8)(p11q13)	*MOZ-TIF2*[77]	AML-M0, M1, M5
t(8;22)(p11;p13)	*MOZ-p300*[78]	AML-M5
-5/del 5q	?*5NCA*[79]	AML, MDS
-7/del 7q		AML, MDS
del 20q		MDS, MPD

Table 1.2. Recurrent cytogenetic abnormalities of myeloid malignancies with cloned oncogenes and disease associations.

1.4.1.1 Translocations affecting CBF.

A critical step in the regulation of the transcription of myeloid genes involves the reversible remodelling of chromatin from an inactive to an active configuration by changing the acetylation status of core histones[80]. Activating proteins bind specific DNA sequences and recruit histone acetyl transferases (HATs) that are responsible for the acetylation of histones and the conversion of DNA to a transcriptionally open form. Conversely, histone

deacetylases (HDACs) repress transcription. One of the most important activators of genes involved in haematological development is the core binding factor (CBF). CBF consists of a complex of an α subunit that binds DNA through a runt homology domain and the β subunit (CBFβ) which facilitates DNA binding. The α subunit can vary but in haematological tissues the protein is called AML1 (also referred to as CBFA2 or RUNX1). CBF recruits the HATs, p300 and CREB-binding protein (CBP) that in turn acetylate histones and activate master gene involved in myeloid development. Several common translocations seen in AML involve *CBF, p300 or CBP*. The AML M2 associated t(8;21) underlies a fusion gene consisting of the runt domain of *AML1* and most of the gene *eight twenty one* or *ETO* (also known as *MTG8*). ETO is able to recruit an HDAC complex and as a consequence of the *AML1-ETO* fusion, CBF is converted to a repressor rather than an activator of myeloid development (reviewed by Downing 1999, Licht 2001[81,82]). *AML 1* is also recurrently involved in translocation with other genes and the partner gene can dramatically influence the type of leukaemia which develops, for example *TEL-AML1* is most commonly seen in 20-25% of childhood ALL (see **1.4.3**).

As a result of the inv(16)(p13q22) *CBFβ* becomes fused with the myosin heavy chain gene *MYH11* a rearrangement that is associated particularly with FAB subtype M4-Eo. CBFβ-MYH11 is thought to sequester AML1 into multimeric complexes where it is unable to perform its function as a transcription regulator[83,84]. The two HATs p300 and CBP are both fusion partners of MLL (see **1.4.2**) and MOZ which its self stimulates AML-1 mediated transcription. The MOZ-CBP fusion protein has been shown to impair the ability of AML-1 to induce differentiation[85].

It is thought that the protein products of several of the CBF related translocations act in a dominant negative fashion, effectively resulting in functional inactivation of both alleles. Dominant negative effects could be mediated through several mechanisms. For example if an abnormal transcription factor complex binds DNA in a more stable fashion than its wild type counterpart, expression of target genes would be competitively inhibited. Alternatively formation of dimeric or oligomeric complexes could result in trafficking of both normal and abnormal proteins to an inappropriate cellular compartment. Dominant negative effects can

mimic homozygous loss of a classical tsg. Accordingly CBF genes have been investigated for evidence of leukaemia related inactivating mutations. As a result of these investigations mutations resulting in either homozygous or heterozygous inactivation of AML1 have been described in cases of AML (discussed further in **1.5**)[86,87].

1.4.1.2 translocations affecting RARA.

A second important group of chimeric genes that interfere with the balance of HAT/HDAC modulated transcription of myeloid differentiation involve the *Retinoic acid receptor α (RARA)*. To date five *RARA* partners have been cloned; *promyelocytic leukaemia* (*PML*), *promyelocytic zinc finger* (*PLZF*), *nucleophosmin* (*NPM*), the *nuclear mitotic apparatus* (*NuMA*) gene and *STAT5b*. Of the five fusion transcripts, which have been studied intensively in mouse models (reviewed by Pandolfi 2001[88]), by far the most common is *PML-RARA*. Like other *RARA* translocations, t(15;17)(q22;q12) producing *PML-RARA*, is associated with APL (AML-M3). However, *PML-RARA* is distinguished from the four variant fusion genes in that it is thought not only to block differentiation through effects on RARA mediated transcription but also to have a tumour suppressor like effect resulting from interference with normal PML function (reviewed by Salomoni and Pandolfi 2002[89]). This dual contribution to tumourigenesis perhaps explains the frequency of *PML-RARA* when compared to the other *RARA* fusion genes.

The normal RARA protein forms a heterodimer with a Retinoid-X-Receptor (RXR-RA) that in the absence of retinoic acid (RA) forms a complex with transcriptional co-repressors. These, in turn, are able to recruit HDACs and repress transcription of RA responsive genes. In the presence of physiological concentrations of RA the RARA-RXR-RA complex binds transcriptional co-activators, in place of the co-repressors, leading to recruitment of HATs and activation of RA responsive genes that include some involved in the terminal differentiation and growth arrest of myeloid cells. PML-RARA-RXR-RA heterodimers, remain complexed with the co-repressors at normal levels of RA resulting in maturation arrest of the myeloid lineage at the promyelocyte stage[90]. The dominant negative effect on transcription mediated by PML-RARA however, appears to be only partly responsible for the contribution of the t(15;17)(q22;q12) to leukemia because mice expressing the *PML-RARA* and *RARA-PML* fusion transcripts develop leukaemia more quickly than those expressing

PML-RARA alone[91]. The mechanism by which RARA-PML contributes to the malignant process is presently unclear but may be related to disruption of normal PML function.

Using immunofluoresence the PML protein was demonstrated to be concentrated in distinct microscopically visible sub-nuclear structures, sometimes known as PML nuclear bodies (PML-NBs)[92]. Typically 10-30 of these structures are found in the inter-chromatin domain with some variation in both number and size during the cell cycle[93]. Formation of the PML-NBs is dependent on the presence of PML but more than 30 other proteins, including p53, pRb, CBP and p300, have also been found to associate with the PML-NBs. In APL blasts, PML-RARA causes the PML-NBs to be disrupted and also to block pRb mediated transcriptional repression of the E2Fs[94]. As discussed in section **1.2** G1-S transition is held in check when hypophosphorylated pRb binds the E2Fs and recruits HDAC complexes, so PML-RARA and or RARA-PML appear not only to block myeloid differentiation but also to remove constraints on cell growth through a second route. Furthermore, UV induced pro-apoptotic post-translational modifications of p53 have been shown to depend on its association with homeodomain interacting protein kinase-2 (HIPK2) and CBP within PML-NBs[95]. Normal PML is also thought to be required for some p53 independent apoptotic pathways such as those stimulated by FAS and TGFβ[341]. It seems likely therefore, that PML has a tumour suppressor (or caretaker) like function that is lost or abrogated as a result of fusion to RARA.

Addition of the drug all-*trans* retinoic acid (ATRA) to cultured PML-RARA positive blasts leads to the reappearance of PML NBs and dissociation of the PML-RARA-RXR co-repressor. Administration of ATRA also restores normal myeloid differentiation in cases of PML. Interestingly inhibitors of HDACs can mimic the effects of ATRA in PML and are being considered a potential treatment option in other cases of AML with maturation block resulting from abnormal HDAC activity[96].

1.4.1.3 Translocations affecting homeobox genes.

The translocations so far discussed modify function of two master regulators of myeloid development, AML1 and RARA. The full complement of AML1 and RARA responsive genes is presently unknown but undoubtedly includes HOX and class II homeobox

transcription factors that operate in a network to maintain blood cell homeostasis[52]. It is therefore unsurprising that several homeobox genes have been found at the break points of leukaemia related translocations. In cases of AML (or CML myeloid blast crisis) *HOXA9, HOXA11, HOXD11* and *HOXD13* have been found fused to the nucleoporin gene (*NUP98*). The fusions all produced an in-frame chimeric gene consisting of an intact DNA binding homeodomain fused to the 5' part of *NUP98* under the control of its strong transcriptional activation domain[66,69,97,98]. A similar fusion gene results from t(1;11)(q23;p15) involving *NUP98* and the class II homeobox gene *PMX1*[68]. Another class II homeobox gene *HLXB9* is recurrently involved in translocation with *TEL* (*ETV6*) in cases of infant AML[75]. The *ETV6-HLXB9* translocation produces an in-frame fusion transcript but wild type *GSH2*, also a class II homeobox gene, was expressed ectopically, as the result of a translocation that interrupted ETV6. A similar position effect was reported by the same authors to result in expression of the cytokine IL3[99]. HOX and related homeobox genes have also been involved in rearrangements with *MLL* or in ALL associated translocations (see **1.4.2** and **1.4.3**).

1.4.1.4 deletions.

Deletions of the long arm of chromosomes 5 and 7 are relatively frequent in cases of MDS and AML and deletions of 20q associated with MDS and the MPDs. The techniques of FISH, southern blot and microsatellite analysis have been used, in successive studies, to narrow down common deleted regions (CDRs) on all three chromosomes[100-103]. In each case more than a single CDR has been defined and mutation or expression analysis of candidate tumour suppressor genes performed on patients carrying deletion of one homologue.

Although a promising candidate tsg, because of its involvement in the regulation of the cell cycle and transcription, no mutations were found in the *ORC5L* gene, mapping to chromosome 7 band q22 and in any of 9 patients tested[104]. The *PIK3CG* gene, encoding a subunit of PI3K gamma, also maps to 7q22 and was considered a good candidate because mice mutated for Pi3k developed haematologic defects. Of 40 patients with leukaemia and deletions of chromosome 7 two were shown to carry an identical missense variation in the N terminal domain of the protein. However, unaffected parents of the two patients and one of 60 control alleles also carried the variant sequence which was therefore considered unlikely to be leukaemia related[105]. A third gene, *HTIFIα* was investigated because its encoded

protein was shown to interact with both RARA and PML. Although *HTIFIα* was deleted in all patients with myeloid malignancy and deletion or translocation of 7q32, its expression was elevated in leukaemias and particularly in those of an immature FAB sub type[106].

Mutation analysis of several candidate tsgs on 5q31 has also been performed. These include *5qNCA, ETF1, CDC23, TTID, RAB6KIFL, PURA and KLHL3*[79,107-112]. Among the tested candidate genes, evidence for mutation of the second allele was found only in *5qNCA* which displayed a THR to ALA change in one cell line bearing a 5q deletion. The potential mutation was not seen in any of 60 normal alleles and furthermore expression of *5qNCA* in a del (5q) cell line resulted in suppression of clonogenic growth. The function of the 5qNCA protein is uncertain but it contained a domain often seen in proteins that regulate chromatin remodelling. Hemizygous loss of *PURA* and *PURB* (located on 7p) have been suggested to have a synergistic tumour suppressor effect because loss of one allele of both genes occurred at a higher rate than would be expected by chance in cases of MDS transformed to AML. The two PUR genes are known to form heterodimers and to affect cell cycle control through interaction with Rb.

No data has yet been presented in support of the candidacy of individual genes on 20q although narrow CDRs containing relatively few genes have been published[103,113,114]. Although little evidence for the existence of recessive tsgs (with the possible exception of *5qNCA*) on chromosomes 5, 7 or 20 has emerged from the extensive studies discussed, heterozygous loss of any of the candidate tsgs might contribute to myeloid malignancy (see **1.5**).

1.4.2 Recurrent cytogenetic abnormalities associated with both myeloid and lymphoid malignancies.

1.4.2.1 The Philadelphia and related Translocations.

The Philadelphia (Ph) translocation [t(9;22)(q34;q21)] as well as being numerically and clinically one of the most important leukemia associated translocations was also the first to be described and so has been studied in depth. At the molecular level several fusion oncogenes result from breaks in different introns of the *BCR* and *ABL* genes (Reviewed by Melo 1996[115]). The most common fusion transcript results from breaks occurring in a 5.8Kb

[major breakpoint cluster region (M-BCR)] between exons 12 and 16 of the *BCR* gene and result in production of a p210 fusion protein. Rearrangements of M-BCR occur in all cases of CML although in approximately 5% the Ph translocation is not detectable by conventional cytogenetic techniques. The Ph translocation is also the commonest rearrangement seen in adult ALL, in some cases breaks occur in M-BCR but more commonly between exons 1 and 2 [the minor breakpoint cluster region (m-BCR)] producing the smaller p190 fusion protein. Both M-BCR and m-BCR translocations are associated with poor outcome in ALL. Alternative rare *BCR-ABL* fusions have been reported; cases of chronic neutrophilic leukaemia have been associated with breakpoints downstream of exon 19 of *BCR* and a 230 Kd fusion protein. Recently cases of CML with breakpoints falling within exon 8 leading to production of a p200 protein have also been characterised[116].

By contrast with the majority of translocations seen in AML, neither partner of the Ph translocation encode a gene involved in the regulation of transcription. The Normal ABL protein (c-Abl) is a tyrosine kinase located predominantly in the nucleus were its primary role is thought to involve the transduction of DNA damage signals and induction of apoptosis (reviewed by Wang 2000 and Blume-Jensen and Hunter 2001[10,117]). Activation of c-Abl is dependent on ATM and its critical target is probably p73 that, on phosphorylation induces cell cycle arrest and apoptosis in part through induction of p53 target genes. The Bcr protein is currently of uncertain function but probably has a role in cellular signalling. Bcr-Abl is localized exclusively in the cytoplasm where it forms autophosphorylated oligomeric complexes that activate pathways normally controlled by growth factor receptor tyrosine kinases (see **1.1.1**)(reviewed by Deininger et al 2000[118]). The self-activating properties of Bcr-Abl depend critically on the presence of 5' oligomerisation domains donated by Bcr.

Although the expanded cell population in CML consists of the granulocytic lineages there is overwhelming evidence to suggest that the *BCR-ABL* fusion originates in a pluripotent stem cell. For this reason CML is often considered to be a stem cell disorder. Consistent with this view is the observation that the blast crisis phase (CML-bc) may be of either myeloid or lymphoid type. In 60-80% of cases transformation to blast crisis is accompanied by acquisition of additional cytogenetic abnormalities. The most common changes are the

appearance of an additional Ph chromosome, trisomy 8 and i(17q)(q10) all of which are found in both myeloid and lymphoid CML-bc[119].

ABL has also been found fused with *ETV6* in rare cases of CML or T-ALL[74]. ETV6-Abl, like Bcr-Abl, is localised to the cytoplasm where the oligomerised chimeric protein is constitutively activated through autophosphorylation. Other receptor and non-receptor tyrosine kinases including PDGFRβ, JAK2, TRKC (NTRK3), ARG (ABL2), FGFR3 and Syk are similarly activated through fusion to ETV6 in various haematological malignancies [72,120-123]. In all cases exons 1-5 of ETV6, including the oligomerisation domain are fused to the 3' part of the partner gene. The Bcr-Abl and ETV6-tyrosine kinase fusion-oncogenes can be functionally related to those encoded by a group of translocations associated with cases of myeloproliferative disorder/myelodysplastic syndrome (MPD/MDS) that involve the *platelet derived growth factor receptor β* gene (*PDGFRβ*) (reviewed by Steer and Cross 2002[124]). Constitutive activation of PDGFRβ in MPD/MDSs results not only from translocation with *ETV6* but also with a growing list of other partner genes including *H4, HIP1, CEV14*, and *Rab5*.

Like *ABL*, *BCR* also has a second, much rarer translocation partner, the *fibroblast growth factor receptor 1* gene (*FGFR1)*[125]. The *BCR-FGFR1* fusion gene occurs in cases of MPD resembling CML but with a distinctive clinical course typically involving transformation to lymphoma. FGFR1 is another example of a receptor tyrosine kinase that becomes self-activating as a result of translocation. Unsurprisingly FGFR1 may be activated through translocation to other partner genes including *ZNF198*, *CEP110* and *FOP* that donate oligomerisation domains. FISH analysis suggests that other rare translocations including t(8;17)(p11;q25), t(8;11)(p11;p15) and t(8;12)(p11;q15) also disrupt *FGFR1*[126].

1.4.2.2 Translocations and other abnormalities affecting MLL.

Translocations of 11q23 occur in AML, MDS, biphenotypic leukaemias and ALL (Reviewed in [56,127,128]). Approximately 40 different translocations of 11q23 have been reported of which about 30 have been cloned and found to involve breaks within an 8.3 kb region between exons 5 and 11 of the Mixed Lineage Leukaemia *(MLL)* gene. The better known, cloned

partner genes are presented in Table 1.3 with their cytogenetic localisation and a brief description of function or functional domains. In addition to gene fusion, two other rare classes of *MLL* mutation have been described; 1) partial amino terminal duplications and 2) deletions of exon 8[129-131].

Like the CBF complex proteins and RARA, MLL functions as a master regulator of transcription. Mice mutated for *MLL* show developmental defects and haematopoietic abnormalities that are accompanied by aberrant patterns of HOX gene expression[132,133]. At its amino terminal end MLL has three AT hooks that are thought to mediate DNA binding and are always retained in the expressed product of the fusion genes. Other domains that appear to make an essential contribution to the transforming properties of MLL fusion proteins are two CxxC motifs that are likely to regulate transcription through DNA methylation and two regions that target MLL to subnuclear domains or speckles[134,135].

The mechanisms by which diverse *MLL* partner genes affect transcription of MLL targets, or contribute to leukaemogenesis in other ways, is currently a matter of debate (for extensive review see Ayton and Cleary 2001[136]). The observation that duplications or deletions within *MLL* can be oncogenic has prompted speculation that the essential leukaemic effect of the translocations is loss of normal MLL function. However, in *in vitro* assays, domains within some of the partner proteins as well as the amino-terminal part of MLL, have been shown to be essential for transformation[137]. Furthermore, the partner genes are always in frame and can influence both the type and severity of disease. About half of the partner genes are nuclear effectors of transcription of which several, including p300 and CBP (see **1.4.1**) are also involved in other oncogenic translocations. The incidence of different *MLL* translocations is also highly variable, those most abundant in myeloid disorders are t(9;11)(p22;q23), t(11;19)(q23;p13.1) and t(11;19)(q23;p13.3) while the t(4;11)(q21;q23) is seen in 60% of infant ALL patients.

Chromosomal locus	Gene[Reference]	Domain/Function
1p32	*AF1p/Eps15*[138]	α-helical coiled coil
1q21	*AF1q*[139]	ATTA motif
3p21	*AF3p21*[140]	SH3 domain
4q21	*AF4*[141]	Ser/Pro-rich, nuclear localisation sequence
5q12	*AF5α*[142]	α-helical coiled coil, GLGF motif
5q31	*AF5q31*[143]	Ser-rich, similar to AF4
6q21	*AF6q21*[144]	Forkhead DNA binding/transcription factor
6q27	*AF6*[145]	α-helical coiled coil, GLGF motif
9p22	*AF9*[146]	Ser/Pro-rich, similar to ENL
10p11.2	*ABI1*[147]	SH3 domain, Homeodomain homologous domain
11q23	*LARG*[148]	Guanine nucleotide exchange factor
16p13	*CBP*[149]	Transcriptional coactivator, HAT
17p13	*GAS7*[150]	Growth arrest specific gene
17q21	*AF17*[151]	Leucine zipper, Zn finger
17q25	*AF17q25*[152]	Septin family
19p13.1	*ELL/MEN*[153]	Pol II transcription elongation factor, p53 interaction
22q11.2	*p300*[154]	Transcriptional coactivator, HAT
Xq13	*AFX1*[155]	Forkhead DNA binding, transcription factor

Table 1.3. Cloned MLL fusion partners; cytogenetic location, gene and gene function or functional domain.

1.4.2.3 Deletions.

Deletions of the p arm of chromosome 17 occur in all haematological malignancies and are particularly associated with transformation of myeloproliferative disorders to acute leukaemia. The deletions result in loss of one copy of the p53 gene and are frequently, but not always, accompanied by inactivating mutations of the second allele[156-159]. As discussed in **1.1.4,** p53 functions include the regulation of apoptosis, cell cycle transition and repair of potentially oncogenic DNA damage. Deletion of the p arm of 17 may be accompanied by duplication of the q arm through formation of an isochromosome 17 i(17)(q10). Currently, it is not known whether the duplication of genes on 17q also contribute to leukaemia. The significance of epigenetic silencing and heterozygous loss of p53 are discussed in **1.5**.

1.4.3 Cytogenetic abnormalities associated with lymphoid malignancies.

Like those found in AML, translocations occurring in lymphoid disease may result in the interruption of transcription factors and production of fusion oncogenes. More typically, though intact proto-oncogenes are brought under strong promoter sequences of antigen receptors as a result of aberrant recombination occurring during the course of *IGH*, *IGL* and *TCR* rearrangement. Hypodiploidy and Hyperdiploidy as well as deletion of specific chromosomal regions are also characteristic of lymphoid malignancies. The more important cytogenetic abnormalities with disease subtype associations and underlying molecular rearrangements, where known, are presented in Tables 1.4, 1.5 and 1.6. (for reviews see[127,128,160]). In the interest of limiting the size of this section and because they have no direct bearing on the subject of this thesis, abnormalities that are restricted to the Lymphomas have not been discussed.

1.4.3.1 Translocations affecting ETV6.

As outlined in **1.4.1** and **1.4.2** rare translocations between *ETV6* and either homeobox genes, IL3 or one of a variety of receptor or non-receptor tyrosine kinases occur in myeloid malignancies. *ETV6* also participates in the most common rearrangement found in childhood ALL, the t(12;21)(p13;q22) that results in production of the *ETV6-AML1* fusion oncogene[161].

The translocation is cytogenetically invisible and can only be detected using FISH or molecular analysis.

The wild type ETV6 protein codes for a widely expressed, sequence specific transcription factor required for normal haematopoiesis. The C terminus DNA binding domain is conserved in the ETS family of proteins. At the N terminus a helix-loop-helix (HLH or pointed) domain mediates protein-protein interactions and homo-dimerisation. Between the ETS and HLH domains is a second protein-protein interacting domain that can repress transcription through recruitment of the co-repressor N-CoR. A second co-repressor (mSin3A) that appears to augment N-CoR mediated repression, can be recruited by the HLH domain[162]. Both N-CoR and mSin3A recruit HDACs and the two probably interact to form a stable repression complex. The HLH domain can also interact with other proteins including UBC10 that conjugates SUMO-1 to *ETV6*. SUMO-1 modified ETV6 localises to nuclear speckles (TEL bodies) in a cell cycle specific fashion[163]. As described in **1.4.1.1**, AML1 is a DNA binding protein that forms part of the CBF complex that can either activate or repress the transcription of genes involved in haematopoietic development through recruitment of co-activators and or co-repressors. As a consequence of the t(12;21) the HLH and N-CoR binding repressive domains of ETV6 are fused to almost all of AML1 including the runt homology DNA binding domain. It is likely that addition of the ETV6 domains converts AML1 from a regulated to an unregulated (repressive) transcription factor, and that this is the major mechanism by which ETV6-AML1 contributes to development of ALL[164]. However, on modification by SUMO1, ETV6-AML1 was observed in the TEL bodies rather than distinctly different nuclear domains occupied by wild type AML1. Abnormal nuclear compartmentalisation of AML1, and its associated proteins, might also influence the course of ETV6-AML positive leukaemia[163].

Tyrosine kinases become self-activating as a result of fusion to ETV6 because dimerisation of the chimeric proteins through ETV6 HLH domains takes place (see **1.4.2.1**). This mechanism clearly differs from ETV6 mediated repression of AML1 transcription targets. By contrast, dimerisation between ETV6-AML1 and wild type ETV6 probably has an anti-leukaemic effect because the translocation is almost invariably accompanied by deletion of

the second *ETV6* allele[165]. Deletions of 12p also occur, albeit very rarely, in cases of childhood ALL in the absence of t(12;21) so ETV6 may have a tumour suppressor function independent of the translocation[166]. However, biallelic loss of *ETV6* has not been reported, raising the possibility that inactivation of one or more other genes on 12p contributes to leukaemia (see **1.4.3.4**).

Other much rarer translocations involving *ETV6* and arising in ALL result in the *ETV6-JAK2* fusion discussed in **1.4.2.1** and *ETV6-PAX5*[167]. *PAX5*, encodes for a transcription factor expressed in early B-cells and is also deregulated as a result of translocation to the *IGH* locus.

Abnormality	**Molecular rearrangement**[Reference]	**Disease sub-type**
t(12;21)(p13;q22)	*ETV6-AML1*[168]	Childhood B-ALL
t(9;12)(p24;p13)	*ETV6-JAK2*[122]	ALL
t(9;12)(q11;p13)	*ETV6-PAX5*[167]	ALL
t(1;19)(q23;p13)	*E2A-PBX1*[169]	B-ALL
t(17;19)(q21;p13)	*E2A-HLF*[170]	B ALL
t(11;18)(q21;q21)	*API2-MLT*[171]	MALT
t(2;5)(p23;q25)	*ALK-NPM*[172]	ALCL

Table 1.4. Recurrent translocations of lymphoid malignancy resulting in the formation of fusion oncogenes. Cloned genes and disease associations. ALCL (anaplastic large-cell lymphoma), MALT (marginal zone lymphoma of mucosa-associated lymphoid tissue type).

1.4.3.2 Translocations affecting E2A.

The t(1;19) resulting in the *E2A-PBX1* fusion oncogene is associated with about 20% of cases of paediatric pre- and pro-B cell ALL. *E2A* encodes a bHLH transcription factor that is

required for immunoglobulin and T-cell receptor rearrangement and later stages of lymphoid development. *PBX1* is a class II homeobox gene encoding a protein that participates in the regulation of cell differentiation through its interaction with HOX proteins. As a result of the t(1;19) the transactivation domain of E2A is joined to the DNA binding domain of PBX1 producing a fusion protein that is thought to have multiple leukaemogenic effects (reviewed by Aspland and Bendall 2001[173]). First, presence of the E2F transactivation domain allows PBX1 to activate its target genes in the absence of HOX coactivators. Secondly E2A is thought to behave as a tsg because it activates the CDK inhibitor p21and mice inactivated for E2A develop thymomas[174,175]. Thirdly the N-terminal domain of PBX1, not present in the E2A-PBX1 fusion protein, contains a nuclear/cytoplasmic localisation signal. Wild type PBX1 is shuttled from nucleus to cytoplasm in a controlled way while E2A-PBX1 is located permanently in the nucleus[176]. The constitutive nuclear localisation of E2A-PBX1 may function to alter expression patterns of HOX/PBX1 target genes.

A second translocation involving E2A [t(17;19)(q22;p13)] is seen in rare cases of adolescent pro-B-cell leukaemia. In this case the partner gene is HLF a gene involved in an evolutionarily conserved cell survival pathway (reviewed in Seidel and Look 2001[177]).

1.4.3.3 Oncogenes deregulated by translocation to antigen receptors.

A diverse selection of genes may be deregulated as a result of translocations that replace constitutive regulatory sequences with those governing the antigen receptors (Table 1.5)(reviewed in [127,160]). The observation that variable length non-template sequences become inserted at the break point junctions of these translocations suggests that ligases associated with normal V(D)J recombination are involved in their production. Illegitimate immunoglobulin gene rearrangements are found in B-cell malignancies and TCR rearrangements in T-cell leukemia/lymphoma. In most cases the deregulated gene is translocated to only one antigen receptor gene and is associated with a specific lineage. A notable exception is the gene *MYC* that may be translocated to *IGH, IGL, IGK* or *TCRA* and is deregulated in several different sub-types of ALL/NHL. Upregulation of *MYC* leads to an imbalance in the ratio of MYC-MAX to MAD-MAX protein complexes that regulate transcription of genes controlling normal lymphoid differentiation, proliferation and

Abnormality	Molecular rearrangement[Reference]	Disease sub-type
t(2;8)(p12;q24)	*IGK-MYC*[178]	B-ALL
t(8;14)(q24;q32)	*MYC-IGH*[179]	B-ALL, DLBCL, BL
t(8;22)(q24;q11)	*IGL-MYC*[178]	B-ALL
t(14;18)(q32;q21)	*IGH-BCL2*[180]	B-ALL, DLBCL, FL
t(11;14)(q13;q32)	*BCL1-IGH*[181]	B-ALL, MCL, CLL, MM
t(14;19)(q32;q13)	*IGH-BCL3*[182]	B-ALL, CLL
t(5;14)(q31;q32)	*IL3-IGH*[183]	B-ALL
t(9;14)(p13;q32)	*PAX5-IGH*[184]	LPL
t(4;14)(p16;q32)	*FGFR-IGH*[185]	MM
t(6;14)(p16;q32)	*MUM1-IGH*[186]	MM
t(1;14)(p22;q32)	*BCL10-IGH*[187]	MALT
t(3;14)(q27;q32)	*BCL6-IGH*[188]	FL,DLBCL
t(10;14)(q24;q32)	*NFKB2-IGH*[189]	DLBCL
t(1;14)(q21;q32)	*MUC1-IGH*[190]	DLBCL
t(1;22)(q21;q11)	*FCGR2B-IGL*[191]	DLBCL
t(8;14)(q24;q11)	*MYC-TCRA*[192]	T-ALL
t(1;14)(p32;q11)	*TAL1-TCRD*[193]	T-ALL
t(7;9)(q35;q34)	*TCRB-TAL2*[194]	T-ALL
t(7;11)(q35;p13)	*TCRB-LMO2*[195]	T-ALL
t(7;19)(q35;p13)	*TCRB-LYL1*[196]	T-ALL
t(10;14)(q24;q11)	*HOX11;TCRD*[197]	T-ALL
t(11;14)(p13;q11)	*LMO2;TCRD*[198]	T-ALL
inv(14)(q11q32)	*TCL1;TCRD*[199]	T-ALL

Table 1.5. Lymphoid malignancy associated translocations of antigen receptor genes. Cloned genes and disease associations. FL (follicular lymphoma), MCL (mantle cell lymphoma), LPL (lymphoplasmacytic lymphoma), DLBCL (diffuse large B-cell lymphoma).

apoptosis (reviewed by Boxer and Dang 2001[200]). Other genes associated with deregulation by IGH in ALL include transcription regulators such as *BCL3* and *PAX5*[184,201], *BCL-2* that plays an anti-apoptotic role through control of cytochrome-c release (described in **1.1.3**) and cell cycle regulators such as *BCL-1*[181] and the cytokine *IL3*[183]. A number of other genes translocated to the immunoglobulin loci contribute to NHL but have not yet been associated with leukaemia.

Deregulated genes that contribute to the development of T-cell malignancy include various transcription factors or co-factors. These include: a homeobox gene, HOX11[342]. The related modifiers of transcription *TAL1 (SCL), TAL2*, *BHLHB1* and *LYL* that are thought to disrupt normal T-cell development by forming heterodimers with, and inactivating, the E2A transcription factor (see **1.4.3.2**)[343,344]. Also the rhombotin genes LMO1 and LMO2 that are thought to participate in the formation of aberrant transcription complexes, possibly in cooperation with TAL1, which disrupt normal T-cell differentiation[345].

1.4.3.3 Deletions and amplifications.

Both deletion and amplification of specific chromosomal regions occur in lymphoid malignancies. Amplification frequently results in up-regulation of the oncogenes that are seen in translocation with antigen receptor loci. Consistently deleted regions are thought to harbour tsgs and in some cases there is strong evidence that loss of function of one or more specific genes influence the course of malignancy.

Deletions of 13q occur frequently in CLL, MM and NHL and also at a lower level in ALL and AML. Because of its role in cell cycle control and unambiguous tsg status in retinoblastoma, the *Rb* gene has been the subject of investigation in these diseases. Results of LOH, deletion mapping by FISH or expression analysis studies suggest that inactivation of Rb contributes to AML and NHL but not CLL or ALL[202-205]. In CLL several groups have pinpointed a more distal region of 13q14 that has been analysed and shown to contain a gene of unknown function and multiple splice forms (BCMS). Sequence analysis of the RNA variants suggested that the BCMS transcripts belonged to a group of non-coding RNAs. As yet a mechanism by which loss of BCMS might contribute to leukaemia has not been

proposed[206,207]. Deletion mapping studies suggest that several different critical regions are lost in MM[208].

Deletions of 9p are common in ALL and a cluster of related genes *p16*^{INK4A}, *p14*^{ARF} and *p15*^{INK4B}are considered tsgs both because of their functional roles as inhibitors of Cyclin/CDK complex formation and activation of p53 (see **1.1.2** and **1.1.3**). *p16*^{INK4A} and *p14*^{ARF} are transcribed from the same locus but in different reading frames and probably modulate each others expression in ways that are as yet poorly understood[209]. The three genes are closely linked on band 9p21 and in ALL deletion of the region occurs in up to 45% of cases. Heterozygous loss of both loci has been reported but more frequently the deletions are biallelic with the *p16*^{INK4A}/*p14*^{ARF} locus most frequently involved[210,211]. Both p16 and p15 may be inactivated through point mutation or promoter methylation (see **1.5**) although homozygous deletions appear to be the favoured mechanism in leukaemia where they confer a poor prognosis[212].

Deletions of 12p are also a common finding in cases of ALL, the region harbours the *ETV6* gene and loss of 12p invariably accompanies the *ETV6-AML1* translocation (see **1.4.3.1**). Loss of 12p also occurs in the absence of t(12;21), but in these cases biallelic inactivation of *ETV6* has not been reported and furthermore one refined deletion map of the region excluded *ETV6*[213]. A CDK inhibitor (p27^{KIP1}) is also located in the frequently deleted region of 12p and has been considered a strong candidate tsg. *p27*^{KIP1} was the most frequently deleted gene in the region as demonstrated by FISH analysis. One of 16 patient showing hemizygous deletion of *p27*^{KIP1} was found to harbour a microdeletion encompassing exon 2 though no inactivating mutations of the second allele were found[214]. In another study a 600kb common deleted region (CDR), bound proximally by *ETV6*, excluded *p27*^{KIP1}. An investigation of methylation changes to CpG islands within the CDR identified de novo methylation at the LRP6 locus in 2 of 22 patients analysed. However, inactivating mutations of LRP6 were not found[215].

Abnormality	Molecular rearrangement	Disease sub-type
Hyperdiploidy		Childhood ALL
Hypodiploidy		ALL
del 13q	*?BCMS, ?RB1*	CLL, MM, NKL
del 9p	$p15^{INK4B}$, $p14^{ARF}/p16^{INK4A}$	ALL
del 12p	*?LRP6, ?TEL, ?*$p27^{KIP1}$	ALL
del 11q	*ATM*	MCL,CLL
del 6q		ALL, DLBCL, CLL, FL, NKL
del 1p		DLBCL
del 1q		DLBCL
del 3p		DLBCL
del 3q		DLBCL
del 7q		SLL, FL
del 10q		FL, DLBCL
amp 2p	*REL*	NHL
amp 8q	*MYC*	NHL
amp 18q	*BCL2,CDK4,*	NHL
amp 12q	*MDM2,GL1*	NHL

Table 1.6. Chromosomal imbalance associated with lymphoid malignancies; chromosome arm, cloned genes and disease association. SLL (small lymphocytic lymphoma)

Deletions and other abnormalities of 11q22-q23 are frequent and confer a poor prognosis in B-cell CLL. Candidate tsgs in the region include *MLL, BCL1* and *ATM*. Of these, *ATM* was found to be the most frequently deleted and Western blot showed 34% of cases of CLL had markedly reduced levels of ATM protein[216]. In a second study 5 of 27 cases of CLL with 11q deletions were shown also to carry acquired mutations of *ATM*, as did three cases without deletion[217]. ATM is a critical component of DSBR (see **1.1.5**) and ataxia telangiectasia

patients develop lymphoproliferative disorders consistent with the evidence that ATM functions as a tsg in CLL.

Deletions of chromosome 6 are discussed in detail in section **1.6** and **1.7**.

1.4.1 Overview of cytogenetic abnormalities in haematological malignancies.

The acute myeloid leukaemias are dominated cytogenetically by translocations that involve genes encoding master regulators of haemopoietic development (*AML1, CBFβ, RARA* and *MLL*) that normally can be positively or negatively acting, depending on the presence of co-regulators. As a result of fusions with multiple partners, the regulated transcription factors become unregulated suppressors of transcription that are also powerful oncogenes. Additional leukaemic effects may be mediated by the reciprocal translocation product and or result from abnormal targeting to nuclear or cytoplasmic domains. A network of homeobox genes that are largely regulated by the master transcription factors play an important part in maintaining blood cell homeostasis. Rare translocations in AML as well as ALL involve deregulation of different homeobox genes.

Translocations seen in MDS, CML and other MPDs are characterised by the involvement of receptor and non-receptor tyrosine kinases that become self-activating as a result of fusion to partner gene oligomerisation domains. Of these BCR-ABL is by far the most common and is diagnostic for CML. The constitutively activated kinases are thought to exert an oncogenic effect by disturbing the normal balance of cellular signals regulating growth and survival. Genes encoding master regulators of transcription (*MLL, AML1, ETV6 and E2A*) also participate in the formation of fusion oncogenes that make an important contribution to infant and childhood ALL. Translocations of *MLL* and *E2A* are also seen in cases of adult ALL but *BCR-ABL* is numerically of much greater importance and carries a particularly poor prognosis in this group of patients. In ALL the lymphomas and multiple myeloma various oncogenes of diverse function are up-regulated as a result of translocations that place them under the control of strong antigen receptor promoters.

Some proto-oncogenes (eg AML1), that as a result of translocation become dominant negative regulators of transcription, are also considered to be tsgs because they may be homozygously or heterozygously inactivated as a result of point mutations. The frequent occurrence of cytogenetically visible deletions of several chromosomal regions suggests that other tsgs also contribute to malignancies of myeloid, lymphoid or stem cell origin. Biallelic inactivation of the multilineage tsg p53 has been found to accompany deletions of 17p in leukaemias of various lineages, other recognised tsgs implicated in haematological malignancy include ATM (11q), Rb (13q), $p15^{INK4B}$,$p16^{INK4A}$/$p14^{ARF}$ (9p) and $p27^{KIP1}$ (12p). Loss of other genes in these regions may also contribute to leukaemia as may currently unrecognised tsgs on chromosomes 5, 7, 20 and 6.

1.5 Classical Tumour Suppressor Genes, haploinsufficiency and Epigenetic effects.

1.5.1 The Classical Tumour suppressor gene model.

In the classical tsg model a single allele of the gene is sufficient to suppress tumour development, i.e. loss of the gene is recessive[218]. This definition stems from early work which established that germ line inactivation of the *RB* gene resulted in cancer prone families. Individuals carrying the *RB* mutations invariably developed retinoblastomas that were shown to be inactivated for both copies of the gene. Mice hemizygous for the *Rb* gene also developed tumours that, even at the microscopic stage, harboured inactivating mutations of the second allele. Sporadic retinoblastomas were also found to show biallelic loss of *RB* function and a similar scenario was established for other genes, for example, the *adenomatous polyposis coli* (*APC*) gene in colon carcinomas[219].

1.5.2 Haploinsufficiency and Malignancy.

As should be clear from the account given of the investigations of candidate tsgs from within leukaemia associated CDRs (**1.4.1.4**, **1.4.2.3** and **1.4.3.3**), the frequency with which inactivating mutations of the second allele are detected, is highly variable. Failure to detect

inactivation of the second allele might occur for several different reasons. 1) Expression of the tsg is only protective against malignancy above a certain threshold or the efficiency of protection is proportional to the level of expression. In this case loss of heterozygosity would be sufficient to promote tumourogenesis but homozygous inactivation might have an additional effect. 2) Inactivation of the second allele could have occurred through mutations in upstream regulatory sequences or epigenetic silencing (see **1.5.3**). 3) Loss of function of a different gene in the same chromosomal region has been selected for and the deletions incidentally encompass the candidate tsg. Recently mouse models have been used to differentiate between the three possible mechanisms.

In a comparison between homozygous p53 knock out, hemizygous p53 knock out and wild type mice, tumours arose most frequently in the homozygous (-/-), at an intermediate level in the heterozygous (+/-) and at a much lower level in the wild type animals. In p53 +/- mice about half of early arising tumours showed homozygous loss of p53 while later arising tumours had predominantly retained the wild type allele. The p53 -/- tumours as well as arising earlier showed greater chromosomal instability and were more aggressive. No evidence for point mutation of the wild type alleles in +/- tumours was found when the coding region was fully sequenced and p53 could be detected on Western blot. Furthermore tests of p53 function were consistent with the presence of a functional allele in the +/- tumours[220].

The tsg *PTEN* is deleted in a range of tumours but the rate of occurrence of inactivating mutations of the second allele is often low. For example 30-40% of invasive breast cancers show loss of 10q23 but inactivating mutations of *PTEN* are found in less than 5%. The offspring of *Pten* +/- mice bred with transgenic adenocarcinoma of mouse prostate (TRAMP) mice were found to develop prostate cancers significantly earlier than Pten wild type TRAMP mice. Inactivating mutations of the second allele were not found in the tumours and furthermore *Pten* -/- TRAMP mice developed tumours at a rate only slightly higher than their heterozygous counterparts[221].

p27^{KIP1} has a function consistent with a tsg role and is frequently deleted in human tumours (see **1.4.3.3**). However, its tsg status was in doubt because inactivating mutations of the second allele have not been reported and familial mutations are unknown. *p27*^{Kip1} +/- mice were shown to develop tumours earlier than wild type controls. None of the tumours showed loss or point mutations of the second allele and the protein was expressed and appropriately targeted to the nucleus[222]. This data strongly supports the suggestion that loss of heterozygosity of *p27*^{KIP1} drives selection of the 12p deletions that are seen in many tumours including lymphoid leukaemias.

Inherited mutations may also contribute to malignancy without loss of the second allele. Families with the rare autosomal dominant disorder known as familial platelet disorder with predisposition to AML (FPD/AML) were recently shown to carry point mutations of *AML1* (*CBFA2)*[87]. As discussed in **1.4**, *AML1* is a component of a transcription complex that is critical to myeloid development and through chromosomal translocation is involved in the formation of several fusion oncogenes. Analysis of leukaemic cells from patients with FPD/AML failed to reveal mutations in the second allele of *AML1* suggesting that this gene can also contribute to leukaemia through haploinsufficiency alone. Interestingly point mutations in the runt domain of *AML1* have been found in approaching 5% of sporadic myeloid malignancy (mostly M0 AML) but in these cases inactivation was biallelic[86].

Overall a scenario is emerging in which the classical tsg model, where homozygous inactivation is absolutely required to overcome a block in the path towards tumourogenesis, represents an extreme that probably applies to only a few genes and maybe limited types of cancer. At the opposite end of the spectrum there may be genes for which heterozygous inactivation results in complete loss of their anti-tumourigenic effects. In this, as yet hypothetical case, deletion or mutation of the second allele would never occur. The majority of tsgs probably behave in a way that is intermediate between the two situations, with a single functional copy of the gene providing some but not complete protection against malignancy. The frequency with which mutations accompany deletion of a tsg would in part depend on how closely its function conforms to one or other of the extreme models. The rate of homozygous inactivation is also expected to depend on the strength of the selective

advantage gained by its loss and the stage of disease at which the advantage is conferred. If loss of a tsg is a late stage event and the selective advantage gained is weak, the frequency of inactivation of the second allele is likely to be low. Conversely more powerful tsgs that arise early in the course of disease will have greater opportunity for biallelic loss.

1.5.3 Epigenetic silencing and malignancy.

In parallel with the acquired changes that in some way modify the germ line genetic code, malignant cells are characterised by major disruptions to the subtle pattern of DNA and chromatin associated protein modifications that regulate normal cellular expression patterns (reviewed by [223]). As discussed in section **1.4** alterations to the acetylation status of histones in gene regulatory regions, are brought about when transcription factors are deregulated as the result of translocation. A second important type of modification involves DNA methylation that may in part also result from the activity of fusion oncogenes[224]. Globally, transformed cells undergo hypomethylation while at the same time CpG islands associated with regulatory regions become densely methylated leading to gene silencing. Methylation changes have been implicated in a diverse range of effects that contribute to the malignant phenotype including; chromosome instability, activation of endogenous parasitic sequences, aneuploidy, loss of imprinting, activation of oncogenes and silencing of tsgs. Depending on the tissue and locus involved promoter methylation may replace deletion or point mutation as a mechanism for tsg silencing. A good example of a genomic region that can be alternatively affected by large deletions, single base pair mutations and CpG island methylation is that containing the $p16^{INK4a}/p14^{ARF}$ and $p15^{INK4b}$ genes. As discussed in **1.4.3.3** these genes are frequently inactivated by deletion and or point mutation in lymphoid malignancies. In T-cell leukaemia/lymphoma the p15 locus was methylated at a rate of 20% while p14 and p16 were methylated at rates of 6 and 3% respectively[225]. In hepatocellular carcinomas hypermethylation of $p14^{ARF}$ was reported to be at a level of 29-46% and of $p16^{INK4a}$ at 25-32%. LOH of the locus was also common among these patients and found to bear a reciprocal relationship to methylation[226].

Imprinted genes are methylated differentially with one parental homologue unmethylated and actively transcribed and the second allele methylated and inactive. In principle

deletion/mutation of only the active allele at an imprinted locus could lead to complete loss of function of a tsg. Loss of imprinting (LOI) appears to be a mechanism by which oncogenes such as IGFII are upregulated in some malignancies including those of the haematopoietic system[227].

In summary cytogenetically visible deletions may unmask inactivating mutations in classical tsgs that are either present in the germ line or somatically acquired. Animal models demonstrate that loss of a single allele of some tsgs is sufficient to promote tumour formation. Tsgs lost as the result of leukaemia associated deletions show great variability in the rate of mutation/deletion of the second allele and it is likely that in some cases a functional protein is produced but at a sub-optimal level. In other cases the second allele may be silenced through an alternative mechanism such as promoter methylation.

1.6 Cytogenetic abnormalities of 6q in haematological malignancies.

Reported cytogenetic abnormalities of both myeloid and lymphoid malignancies include sporadic and rare recurrent translocations involving the long arm of chromosome 6. Four oncogene partners have so far been cloned from 6q translocations, others are still uncharacterised at the molecular level. Among lymphoid malignancies rearrangements that result in apparent loss of 6q material are numerically of much greater importance than are the balanced translocations. Deletions, unbalanced translocations and trisomy of chromosome 6 also occur in cases of myeloid malignancy but at a lower frequency. As yet, no tsg has been identified on 6q.

1.6.1 Balanced translocations of 6q.

Two translocations involving *MLL,* t(6;11)(q27;q23) and t(6;11)(q21;q23) result in the *MLL-AF6* and *MLL-AF6q21* chimeric oncogenes, respectively. *MLL-AF6* accounts for about 5-6% of all *MLL* translocations and although chiefly associated with AML M4 and M5 has also

been found in cases of ALL[228]. Only a few cases of *MLL-AF6q21* have so far been described though there is an apparent association with therapy-related AML[144,229]. As discussed in **1.4.2** MLL is a highly promiscuous oncogene partner that as a result of rearrangement always maintains its DNA binding domain. As a consequence of the rearrangements the transcriptional regulation of MLL targets, that include genes involved in haematopoietic development, is disrupted. However, additional leukaemogenic effects may be mediated through the partner genes that can influence disease type and prognosis.

AF6 encodes a RAS binding protein that as a result of fusion to *MLL* is moved from its wild-type cytoplasmic location to the nucleus[230]. Since *RAS* itself is an important oncogene encoding a protein that transduces growth and survival signals, loss of normal AF6 function resulting from abnormal cellular localisation, may well influence the phenotype of t(6;11)(q27;q23) positive leukaemias. In addition AF6 was recently shown to bind LMO2, a LIM only protein known to act as a bridging molecule between different components of the haematopoietic gene regulatory protein complexes[231]. Thus as a result of translocation AF6 may recruit regulatory complexes to the promoters of MLL target genes.

AF6q21 encodes a member of a family of proteins that have in common a forkhead DNA binding domain. Among the forkhead family members *AF6q21* showed highest homology to two other oncogene partners *AFX* and *FHKR*. *AFX* is also a an ALL oncogene partner and *FHKR* is found fused to two related transcription factors *PAX3* and *PAX7* in alveolar rhabdomyosarcomas[155,232,233]. On the basis of their functional domain content, the forkhead genes are considered to be transcription regulators. In haematopoietc tissues the forkhead family members are expressed in a lineage-restricted manner although their specific functions are currently unknown. Formation of the oncogenic fusion genes result from breaks occurring in the first intron of the forkhead gene in each case, suggesting that one or more common domains make a critical contribution to transformation.

Three of the related constitutively activated tyrosine kinase fusion oncogenes (introduced in **1.4.2.1**) involve the fibroblast growth factor receptor (*FGFR1)* gene positioned on the short arm of chromosome 8. As a consequence of t(6;8)(q27;p11) the *FGFR1* oncogene partner

(*FOP*) becomes fused to *FGFR1* in rare cases of MPD. *FOP* encodes a widely expressed and evolutionarily conserved protein with multiple splice forms. The function of FOP is unknown but the presence of novel leucine-rich repeats suggests that it can participate in protein-protein interactions. The *FGFR1* gene encodes one of four tyrosine kinase receptors that transduce growth, survival and differentiation signals on binding members of the fibroblast growth factor family. As a result of t(6;8) the intracellular tyrosine kinase domain of FGFR1 becomes joined to the N-terminus leucine rich region of FOP that is assumed to mediate oligomerisation of the chimeric gene[234]. Based on the known activity of related oncogenic fusion proteins (such as Bcr-Abl or ZNF198-FGFR1) it is likely that oligomerisation of FOP-FGFR1 results in a constitutively phosphorylated tyrosine kinase that activates FGFR1 downstream targets and stimulates haematopoietic stem cell proliferation. In the case of the t(6;8) originally cloned, fusion between exon 6 of *FOP* and exon 9 of *FGFR1* occurred and both *FOP-FGFR1* and *FGFR1-FOP* transcripts were expressed. A leukaemogenic role for the reciprocal FGFR1-FOP fusion protein has not been postulated. Two further cases of t(6;8), including patient 34 analysed by FISH in this study, have since been cloned and shown to involve fusion of exon 9 of *FGFR1* but exons 5 and 7 of *FOP*[126]. Analysis of a larger group of patients would be required to determine whether the position of break points in *FOP* influence the course of leukaemia in any way.

One further gene on 6q has been implicated in leukaemia as a result of analysis of a t(6;12) in a cell line established from a case of childhood B-cell precursor ALL. Cloning of the gene revealed that fusion between *ETV6* and a novel gene (*six twelve leukaemia* or *STL)* had taken place[235]. *ETV6* is a proto-oncogene that can contribute to the generation of haematological malignancies of different lineages through translocation to multiple partner genes (see **1.4**). Leukaemogenic mechanisms resulting from *ETV6* translocations include ectopic expression through position effect and the formation of chimeric genes that are either deregulated transcription factors or self-activating tyrosine kinases. The significance of the involvement of *STL* in this rare, if not unique, translocation was difficult to gauge from sequence and expression data. The *ETV6-STL* transcript was relatively abundant but gave rise to only a very short open reading frame that excluded the critical HLH and ETS domains of *ETV6*, and coded for only 14 amino acids from *STL*. The *STL-ETV6* transcript could not be isolated from

the cell line c-DNA and would have been transcribed under control of the *STL* promoter. Northern analysis suggested that *STL* was transcribed only at a low level in haematopoietic cells. An important consequence of the translocation may have been loss of *ETV6* function because a deletion of 12p that resulted in loss of the second allele was detected in the cell line. Of interest, in relation to this study, was the fact that the t(6;12) was only recognised after FISH analysis when the break point on 6 was assigned to q23.

Translocations between 6 and 12 with a 6q23 break point have not been reported in cases of leukaemia though a rare but apparently recurrent t(6;12)(q21;p13) was associated with childhood ALL and an early pre-B immunophenotype[346]. In these cases no molecular analysis was performed so it is possible that the break point had been mis-assigned and that these cases also involved *STL*. Two cases of infant acute basophilic leukaemia were reported to have a t(X;6)(p11;q23) as the sole karyotypic abnormality[236]. A third case of t(X;6) with identical break points was associated with acute megakaryoblastic leukaemia[237]. Though evidently rare the t(X;6) seems also to be a recurrent rearrangement though the break points have not been cloned in any of these cases. A second t(X;6) with a break point at 6q15-16 was reported in three cases of congenital or infant leukaemia[238].

In addition to the rare recurrent but uncloned translocations sporadic translocations involving 6q have been reported. In one of the sporadic rearrangements a t(2;6), found in a case of biphenotypic leukaemia, was also assigned a 6q23 break point[239]. Individual reports of other translocations that so far remain unique include a t(6;10)(q27;q11) in a case of idiopathic myelofibrosis and a t(6;8)(q25;q22) in T-cell blast crisis of Ph+ CML[240,241]. Further examples of sporadic balanced rearrangements of 6q that had been included in large cytogenetic studies can be found on searching the Mitelman database of published chromosome aberrations in cancer at (http://cgap.nci.nih.gov/ Chromosomes/Mitelman). These included translocations, paracentric and pericentric inversions and insertions and were in no case the sole cytogenetic abnormality. Twelve of the additional sporadic 6q abnormalities were associated with myeloid and 7 with lymphoid leukaemias. Of myeloid associated abnormalities 5 involved 6q27, other bands were not preferentially involved.

1.6.2 Unbalanced rearrangements of 6q.

Cytogenetically detectable deletions of 6q [del(6q)] are common in both B and T ALL of childhood and adults, CLL, MM and NHL, they may also be found in myeloid malignancies but at a much lower frequency. In several large cytogenetic studies, 4-13% of cases of ALL and 17-23% of NHLs have been assigned deletions of 6q[242-249]. In childhood ALL loss of 6q was found to be associated with abnormalities of either 12p or 9p[250]. In other cases del 6q co-existed with deletions of 12p suggestive for involvement of the other homologue in t(12;21). Loss of a tsg on 6q may therefore be selected preferentially against a genetic background that includes the *ETV6-AML1* fusion or loss of CDK inhibitors.

Morphology of ALL karyotypes is poor and the banding pattern of 6q not highly distinctive, factors that may account for the wide variety of break points assigned to 6q deletions[251]. Despite these difficulties it has been claimed that all 6q deletions seen in ALL involve the band q21 suggesting that a tsg may be located in this region[250]. Early reports that 6q deletions are associated with a poor outcome in ALL have not been substantiated by later studies[252,253].

1.7 FISH and molecular studies of 6q deletions in Leukaemia.

With the aim of identifying a tsg several groups have attempted to establish a common deletion region (CDR) (also often referred to as region of minimal deletion [RMD]) on 6q, using either FISH or the molecular technique of microsatellite analysis. Studies of haematological malignancies, focusing on chromosome 6, have been restricted to the lymphoid malignancies. Some groups analysed both leukaemias and NHL while others have chosen to concentrate on a disease sub-type. Published FISH studies have made use of YAC clones containing sequences of genetic markers or genes mapped to 6q. An early FISH study defined 2 CDRs. The proximal region, common to both ALL and NHL were bounded centromerically by the gene *M6P1* (6q14) and distally by the marker D6S246 (6q21). A second CDR in the q23-q telomere region was restricted to lymphomas and based on the observation of non-contiguous deletions in two patients[254]. In a follow up study from the

same group further non-contiguous deletions in cases of lymphoma confirming the distal CDR. Proximally two ALL and two lymphoma patients defined a narrow CDR between two markers at band 6q21, D6S447 (proximal marker) and D6S246 (distal marker)[255]. Further YACs positioned between these two markers were isolated and used to investigate 21 patients with ALL and cytogenetic deletions of 6q in a study conducted in this department[256]. We found five patients that retained the marker D6S447 but were deleted for more proximal markers. Two patients while retaining D6S447 were deleted for the more distal YAC 36CB11 (containing CD24). Furthermore, one of the patients retained YAC 860f10 that was mapped distal to D6S447. An uncloned gap, that on the basis of interphase FISH we estimated to be approximately 1 Mb in size, existed between YACs 860F10 and 36CB11. We concluded that either 1) expression of a large gene, positioned within the uncloned gap, was affected by both proximal and distal deletions or 2) two discrete and independent regions of deletion in the 6q16-q21 region existed. One case included in the study (patient 21) showed evidence for clonal evolution from a small to a large deletion. The smaller deletion was situated proximal to D6S447 and the larger, detected in a smaller proportion of metaphase cells, extended distally to include D6S302 (band 6q22). The pattern of deletion seen in this case was consistent with loss of the more proximal of our two regions being an earlier stage event in ALL than loss of the more distal region. Deleted and retained probes for critical patients used to define CDRs from the 3 FISH studies are presented diagrammatically in **Fig 1.2**.

One further FISH study and a further four reporting on LOH of 6q markers in ALL samples, have been published[257-261]. The position of the CDRs, derived from these studies, in relation to each other as well as the data obtained in the present study, are presented more extensively in chapter 3 (Fig **3.7**). In one case two CDRs were obtained making a total of six. All six of the published CDRs overlapped with the more proximal of the two independent regions we identified, two also overlapped with the more distal of our regions. It should be emphasised that in three of the five studies not all patients fell within the published CDR and that there were some differences in the disease sub-types, included in the studies. The relationship between the published CDRs is discussed further with respect to data obtained in the current study in chapters 3 and 4.

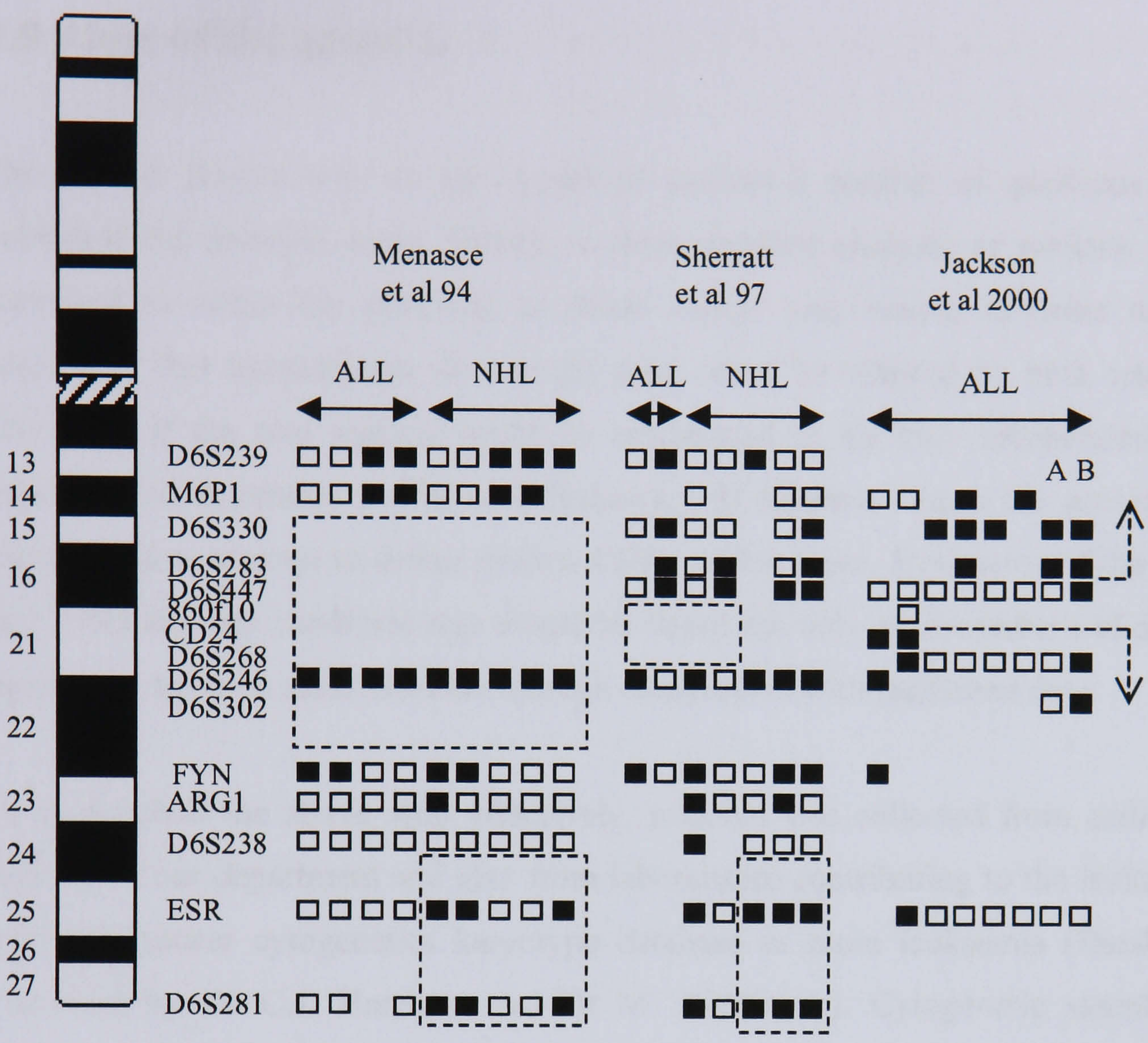


Fig 1.2 Informative patients from three studies in which metaphase cells from cases of lymphoid malignancy with deletions of 6q were analysed by FISH with YACs. Black boxes indicate that the probe was deleted, white boxes indicate that the probe was retained. Common deleted regions (CDRs) defined in each study are indicated by broken lines. In the first two studies loss of the region telomeric to D6S238 was associated with cases of NHL while a more proximal region was common to both NHL and ALL. In the most recent of the studies cases of ALL only were analysed using newly identified YACs from within the earlier defined proximal CDR. Two independent ALL associated CDRs were identified as a result of this study, one proximal to D6S447 and the second distal to YAC 860f10. Clonal evolution from a small (A) to a large (B) 6q deletion appeared to have Taken place in one patient analysed in the final study.

1.9 Aims of the project.

The present project was set up in part to address a number of questions posed by our published 6q deletion study. Firstly, a more detailed analysis of patients with deletions restricted to either the proximal or distal region was needed in order to evaluate the possibility that transcription of a single gene could be affected by both sets of deletions. Secondly, if the two regions could be established to be truly independent it would be important to determine the relative frequency of deletion within the proximal and distal regions and to attempt to define narrow CDRs within them. Evaluation of the importance of the CDRs and any candidate tsgs would be based not only on the pattern of deletion among patients in our own study but also through comparison with published data.

To accomplish the above aims effectively, material was collected from additional patients referred to our department and also from laboratories contributing to the leukaemia research fund UK cancer cytogenetics karyotype database in acute leukaemia (Database) (kindly requested by Dr C.J. Harrison and Dr M. Martineau). Cytogenetic samples from ALL patients and also a small number of AML patients, with cytogenetically detectable deletions of 6q, were obtained. Additional cases of both ALL and AML reported to bear balanced translocations or inversions of 6q were also included in the pool of samples to be analysed.

The YAC probes previously used for FISH analysis of 6q abnormalities were large in size and hence of limited use for the resolution of chromosomal breakpoints. YACs are also prone to recombination and technically difficult to use. Since contigs of PAC and BAC probes now cover a large part of the genome it was decided to establish a panel of these smaller, more robust probes for the analysis of leukaemia associated abnormalities of 6q.

In summary the aims of the project were:

1) To establish a panel of PAC and BAC FISH probes distributed between 6q11 and the 6q telomere.

2) To map the position of breakpoints in cases of acute leukaemia with cytogenetically detectable deletions or balanced rearrangements of 6q, using the panel of FISH probes.

3) To identify one or more CDRs that would then be mapped in greater detail with further PACs and BACs.

4) To identify and assess, by comparison with published data and ultimately using molecular techniques, candidate tsgs from within the CDR/CDRs.

5) To identify common 6q break points among the cases with balanced rearrangements.

6) To map in detail by FISH any common 6q breakpoints identified.

7) To assess the involvement of known proto-oncogenes on partner chromosomes of the 6q translocations.

8) To determine the sequence, at the breakpoints of 6q translocations, found to be either recurrent or to disrupt genes known to be involved in malignancy.

Chapter 2 (Materials and Methods)

Details of the preparation of materials, where not described in the text of this chapter, can be found in appendix 1. All oligonucleotide sequences are presented in Appendix 2.

2.1 Patients' material.

Fixed cell suspensions from bone marrow samples of patients with ALL and AML as prepared for cytogenetic analysis with accompanying karyotypes were obtained from the Department of Haematology, Royal Free and University College School of Medicine. Further ALL samples were obtained from laboratories of the United Kingdom Cancer Cytogenetics Group (UKCCG) on request through the Database. Slides were made (as described in **2.2** below) and stored at -20°C for future analysis by FISH.

2.2 Cell lines.

Cell lines RPMI 8402, Molt 4 and Peer were obtained from the German National Tissue Culture collection (DSMZ). Cells were grown in RPMI-1640medium (Sigma cat # R8758) with 10% batch tested foetal calf serum, penicillin 1mg/ml and streptomycin 1mg/ml in 50ml plastic tissue culture flasks. After overnight incubation with 40μg/ml colcemid (Gibco cat #15210-040) 10 ml of cells were transferred to 15ml centrifuge tubes and pelleted by spinning at 1,600 rpm for 10 minutes. The supernatant was discarded and the cells re-suspended in 10ml 0.075 M KCl before incubating at 37^0C for 10 minutes. The cells were again centrifuged at 1,600 rpm for 10 minutes and the supernatant removed leaving approximately 1ml to resuspend the cells in. After resuspending the cells, 10 ml of ice cold freshly prepared 3:1 methanol/acetic acid fixative was added dropwise while gently agitating the tube. The cells were pelleted and resuspended in fixative twice more, the second time adding only enough fixative to create a milky solution.

Cytogenetic preparations were made by running a drop of cell suspension, followed by a drop of fixative, down a dampened slide. Slides for FISH analysis were sealed in plastic and

stored at -20^{0}C. Slides for G-banded analysis were baked on a hot plate at 60^{0}C overnight, dipped briefly in 1-2% trypsin in PBS then washed in PBS. Slides were then washed in pH 6.8 'Gurr' buffer (Gibco BRL cat # 10582-013) then stained in 20% Leishmans solution in pH 6.8 buffer. After drying and coverslipping the G-banded slides were analysed to confirm the presence of del(6)(q). Further slides were made and stored at -20°C for future analysis by FISH.

2.3 Database Searches.

Clones containing specific markers were identified by searching the chromosome 6 ACEDB database, available through a link at the web site http://www.sanger.ac.uk /HGP/Chr6/. PACs positioned onto contigs, in the ACEDB database, are assigned 6-fpc values for left hand (centromeric) and right hand (telomeric) ends that identified their positions relative to other probes on chromosome 6. The 6-fpc values could therefore be used as a measure of the relative position of all chromosome 6 PACs and BACs in the database although the size of remaining gaps between contigs were necessarily estimated. Sets of probes used for more detailed secondary FISH analysis were obtained by selecting PACs or BACs with fpc numbers intermediate in value between pairs of probes in the primary panel. 6-fpc values quoted for PACs and BACs obtained through HGMP throughout this thesis were accurate at the time of analysis but may be altered in future versions of the chromosome 6 physical map.

FISH analysis of patient and cell line material identified several regions of 6q likely to harbor genes that make some contribution to leukaemia. The regions were contained within contigs derived from mixtures of fully and partially sequenced clones. Fully sequenced clones were viewed in the 6 ACEDB Database where exons of known or predicted genes, positioned on the clones, are presented in graphical form. Relatively large regions of the genome were also scanned rapidly for the presence of known genes using ENSEMBLE, a graphical database run jointly by the European Bioinformatics Institute and the Sanger center at http://www.ensembl.org. Detailed positioning of exons genetic markers and ESTs was carried out using the BLAST search facility of the National Centre for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov.

2.4. Preparation of DNA Probes for FISH analysis.

2.4.1 Large-scale preparation of DNA from PAC and BAC clones (maxiprep)

PAC (from libraries RPCI1, and 4) and BAC (from library RPCI11) clones positioned on chromosome 6 by the human Genome Mapping Project (HGMP) were obtained on request through the UK HGMP resource Centre, Hinxton, Cambridge (http://www.hgmp.mrc.ac.uk). The clone P1-3742 (containing *CCNC*) was kindly supplied by Dr J.M. Lahti, Department of tumour cell biology, St. Jude Children Research Hospital, USA. Clones were streaked onto LB agar plates containing appropriate antibiotic (P1-3742, RPC1, and 4 kanamycin 25µg/ml, RPC11 ampicillin 100 µg/ml), and placed in a 37^0C incubator for 16-48 hours. Single colonies were picked and grown overnight in 50 ml 2xTY containing the appropriate antibiotic.

600 µl of the overnight culture was used to prepare a 15% glycerol stock for storage at -70^0C. The remainder of the culture was transferred to a 50ml Falcon tube and spun in a MSE mistral 2000 centrifuge at 3,000 rpm for 15 minutes. The supernatant was discarded and the cell pellet resuspended by vortexing in 5ml of cold GTE. Following a 10 minute incubation at room temperature the cells were lysed by addition of 10ml of freshly prepared 0.2M NaOH/1% SDS. After mixing and incubating for 5 minutes at room temperature, bacterial DNA, proteins and lipids were precipitated by adding 5ml of 3M Na Acetate (pH 4.8). The contents of the Falcon tube was shaken vigorously and incubated on ice for 15 minutes to 1 hour before centrifuging at 3,000 rpm for 15 minutes. The supernatant was strained through gauze into a fresh Falcon tube. PAC or BAC DNA was then precipitated, by adding an equal volume of isopropanol and incubating on ice for 5-10 minutes. The DNA was pelleted, by centrifuging as before, then resuspended in 2ml of TE. High molecular weight RNA was precipitated by adding 2 ml of 5M LiCl to the suspension and incubated on ice for 5 minutes. The tube was then centrifuged at 3,000 rpm for 5-10 minutes and the supernatant transferred to a fresh Falcon tube. DNA was again precipitated by adding 2 volumes of ethanol (kept at -20^0C), and incubating on ice for 5 minutes. After again centrifuging at 3,000 rpm for 5-10

minutes and discarding the supernatant, the pellet was dissolved in 1ml of TE. To degrade remaining RNA, the tube was placed in a 37^{0}C water bath for 15 minutes after addition of 4µl of 10mg/ml DNase free RNase. DNA was recovered by transferring the solution to a 2ml microcentrifuge tube, adding 500µl 2.5M NaCl/20% polyethylene glycol and incubating on ice for 5 minutes. After centrifuging at 13,000 rpm for 5 minutes the supernatant was discarded and the pellet dissolved in 500µl TE. The DNA solution was cleaned twice with PCIA and once with chloroform/isoamyl alcohol (24:1; v:v) then precipitated by adding 0.1x 3M Na Acetate and 2x ethanol and storing over night at -70^{0}C. The DNA was pelleted by centrifuging at 13,000 rpm for 10 minutes, washed once with 70% ethanol and, after removing the supernatant, allowed to dry at room temperature. The dried pellet was dissolved in 100-200µl TE. Concentrations of DNA were determined and purity checked by measuring absorbance at 260 and 280 nm. DNA solutions were stored at -70^{0}C.

2.4.2 Preparation of Yeast Artificial Chromosome (YAC) DNA.

YAC clones where obtained on request from the UK HGMP resource center, Hinxton Hall Cambridge, streaked onto AHC agar plates and incubated overnight at 30^{0}C. Individual colonies were then picked and used to inoculate 10ml AHC medium in 25 ml plastic universal tubes. The cultures were grown in a shaking incubator at 37^{0}C overnight and cells pelleted by centrifuging for 10 minutes at 3,000 rpm in a MSE Microcentaur bench top centrifuge. After discarding the supernatant the pelleted cells were washed with 500µl distilled H_2O and transferred to 1.5 ml microcentrifuge tubes. The cells were again pelleted by centrifuging at 13,000 rpm for 30 seconds and after discarding the supernatant resuspended in 200µl GDIS solution. Tubes were then vortexed continuously for 5 minutes after adding 0.35g of acid washed glass beads (Sigma cat # G-1152) and 200µl phenol (Sigma cat # T/P633/05). 200µl distilled H_2O was then added to the suspensions before centrifuging at 13,000 rpm for 4 minutes. The aqueous layer was carefully transferred to a second eppendorf tube and treated with 5µl 10mg/ml DNase free RNase A (Sigma) for 20 minutes at 37^{0}C. The DNA was then cleaned by extracting twice with PCIA and once with chloroform/isoamyl alcohol (24:1;v:v). Cleaned DNA was precipitated with 0.1x 3.0M NaAc and 2x Ethanol at -70^{0}C overnight. Tubes were then centrifugation at 13,000 rpm for 10

minutes, the supernatant discarded and DNA pellets washed with 70% ethanol. After washing, the pellets were dried at room temperature and re-suspended in 20-100µl TE depending on the pellet size. An estimate of the concentration of DNA was made by running 5µl aliquots on a 50 ml 0.8% agarose gel with 100-500µg uncut phage λ.

2.4.3 Preparation of fluorescence labeled DNA probes.

For two colour FISH experiments probes were labelled by incorporation of Spectrum Green or Spectrum Red conjugated d-UTP (Vysis cat # 30-803200 and 30-803400) using a nick translocation kit (Vysis cat # 32-801024). Individual labelling reactions containing the following were set up in 1.5ml microcentrifuge tubes on ice; xµl (1µg) probe DNA, (17.5 – x) µl nuclease free H_20, 2.5µl 0.2mM SpectrumGreen or SpectrumRed dUTP, 5µl 0.1mM dTTP, 10µl dNTP mix (0.1mM each of dATP, dCTP and dGTP), 5µl Nick translation buffer, 10µl Nick translation enzyme (a mixture of DNA polymerase I and DNase I). Reactions were mixed by vortexing and incubated for 14 hours in a water bath at 15^0C in the dark. Labelled probes were precipitated overnight at -70^0C with 50µg Cot 1 DNA, 50µg salmon sperm DNA, 50µg tRNA, 0.1 x volume 3M NaAc and 2.5 x volume EtOH. After spinning for 10 minutes at 13,000 rpm in a microcentrifuge the supernatant was discarded. Probes were then washed with 500µl 70% EtOH, centrifuged for 5 minutes and after removing supernatant by pipette allowed to dry at room temperature. Dried probes were resuspended at a concentration of 20ng/µl in hybridisation buffer (Applygene Oncor, cat # S1390-1) and stored at -20^0C in the dark. All probes where hybridised to normal metaphases obtained from PHA stimulated peripheral blood to check for the presence of red or green signals on the long arm of chromosome 6.

For three-colour FISH analysis a third fluorescently labelled dNTP, Alexa 532-5-dUTP (Molecular probes) was incorporated by nick translation as for Spectrum Green or Red.

2.5 FISH procedure and analysis.

2.5.1 Pre-treatment of cytogenetic preparations.

After removing from -20°C slides for FISH analysis were immersed in a freshly prepared 3:1 methanol:acetic acid fixative on ice for 1 hour, dried then treated with 100µl 0.01% pepsin (manufacturer cat #) at room temperature under a coverslip. Slides were placed in a coplin jar containing 2xSSC at 60°C for 1 hour then dehydrated through an ethanol series (70%, 90% and 100%), excess ethanol was allowed to dry at room temperature. Slides to be reused after a previous FISH experiment (re-FISH) where soaked in PBS for 1 hour after first removing nail varnish. Coverslips were then carefully removed before placing slides in fresh ice-cold fixative for 1 hour and then dehydrating as above.

2.5.2 Hybridisation using single locus probes.

2µl of each probe to be used were mixed and made up to a total volume of 5-6µl with hybridisation buffer. The probe mixture was then placed onto the slide and covered with a 22 x 11mm plastic coverslip and after removing air bubbles sealed with rubber cement. Target DNA and probes were then denatured simultaneously by placing the slides on a 70°C hot plate for 2 minutes. Hybridisation of probes to their complementary sequences was achieved by placing slides in a sealed moist chamber for 24-72 hours at 37°C in the dark. Following hybridisation, coverslips were removed and the slides washed once in 0.4xSSC for 3 minutes at 70°C and 3 times for 2 minutes at room temperature in 4xSSC + 0.05% Tween 20 (BDH cat # 663684B). Slides were then coverslipped with 10µl vectorshield antifade solution (Vector Laboratories, Burlington, CA. cat # H-1000) containing 0.2ng/µl DAPI. Before analysis the edges of the coverslips were sealed with nail varnish. Care was taken to minimise exposure of the fluorescent probes to light throughout the procedure.

2.5.3 Hybridisation of whole chromosome paint (WCP) probes.

WCPs derived from flow sorted chromosomes and pre-labelled with Spectrum Green or Spectrum Red were obtained from Cambio Ltd, Cambridge UK. 1µl of each WPC to be used was added to a microcentrifuge tube, made up to a total volume of 6µl with the manufacturers hybridisation buffer and mixed by vortexing. Hybridisation to slide bound chromosomes was performed as described in **2.7.2**. Following hybridisation slides were

washed in 0.5xSSC at 72^{0}C for 5 minutes then 3 times for 2 minutes at room temperature in 4xSSC + 0.05% Tween 20. Slides were mounted as for single locus probes.

2.5.4 Analysis of FISH preparations.

Visualisation of FISH signals was performed on an Axioplan fluorescence microscope (Karl Zeiss, Germany) equipped with appropriate filters (Chroma Technology) and MacProbe software (Applied Imaging International, UK). For analysis of leukaemic samples single locus probes were hybridised in pairs labelled with Spectrum Red and Spectrum Green. Probes labelled in Spectrum Red were either from the 6q centromeric region (RPC1-71H19) or the 6q telomeric region (RCP1-167A14 or RCP1-57M24). A primary panel of 25 Spectrum Green labelled probes positioned on Sanger centre PAC/BAC chromosome 6 contigs between the centromeric and telomeric markers were used for the initial analysis. Analysis was restricted to metaphase cells and signals defined by the appearance of pairs of closely aligned red or green dots, one on each of the two sister chromatids of a chromosome. A metaphase cell was scored only if 2 clearly positive red and or green signals were visible (or 3 or 4 signals in the case of clones trisomic/tetrasomic for chromosome 6). A cell was scored as deleted for a probe if either a red or green signal only was seen on a chromosome and the missing signal not present on any other chromosome (Fig **2.1**). For each cell scored the retained probe acted as an internal positive control for hybridisation efficiency and the marked chromosome was examined to ensure that a signal had not been obscured by overlap with a second chromosome. Clonal deletion was defined by the loss of signal in a minimum of 5 metaphase cells and in not fewer than 20% of the total cells observed.

If red and green signals were seen on two different chromosomes the cell was scored as having a translocation of 6q with a break point between the two probes (Fig **2.2**). A case was defined as having a clonal translocation of 6q if red and green signals were seen on different chromosomes in a minimum of 3 cells and in not fewer than 20% of the cells observed.

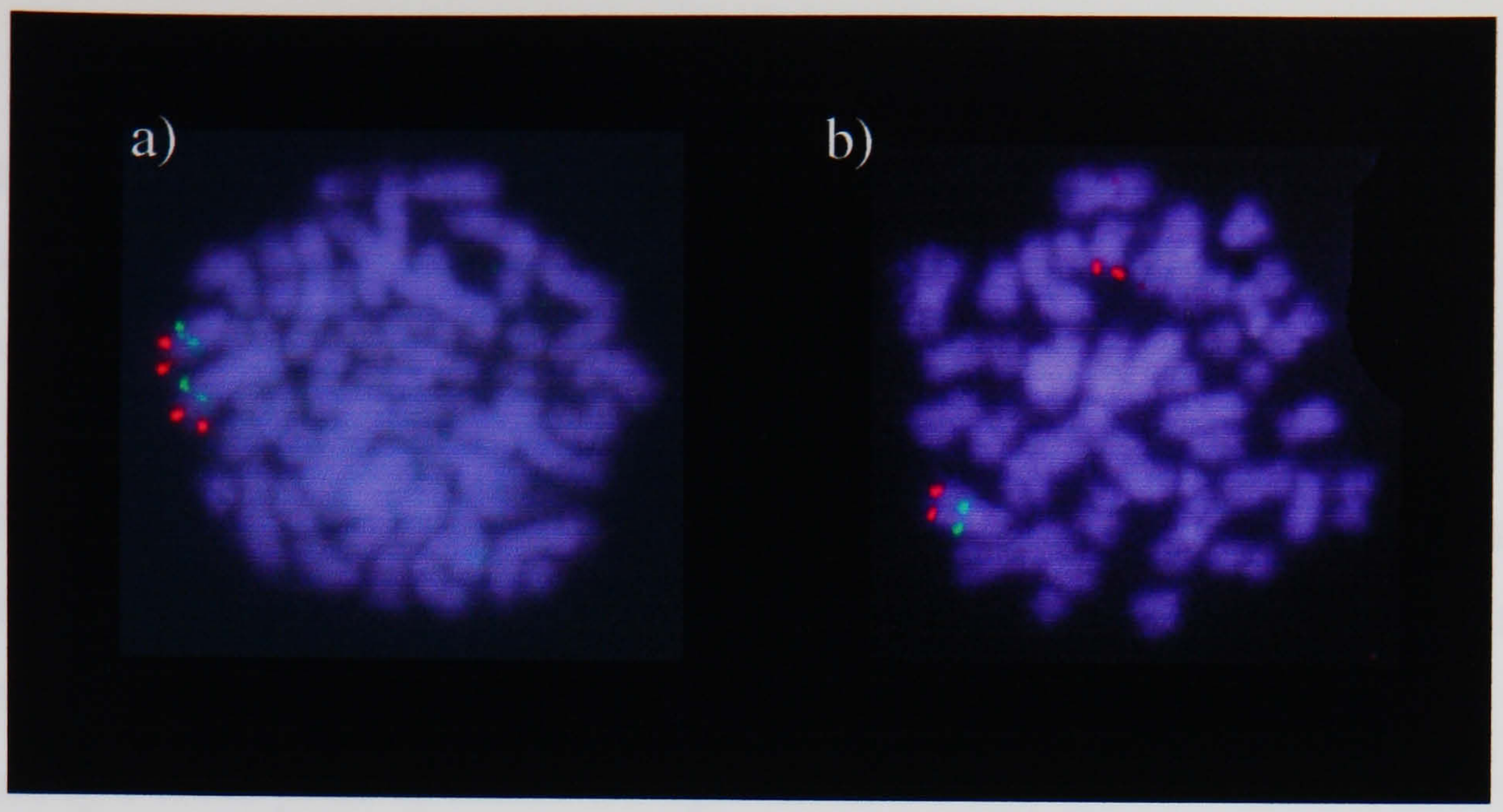


Fig 2.1 Example of FISH probes hybridised to metaphase cells from an ALL patient(Number 2) with del(6q) a) Pairs of red and green signals are present on both the del (6q) and the normal chromosome 6 (probe retained). b) Red and green signals mark the normal chromosome 6 but red signals only are seen on the del(6q) (probe deleted).

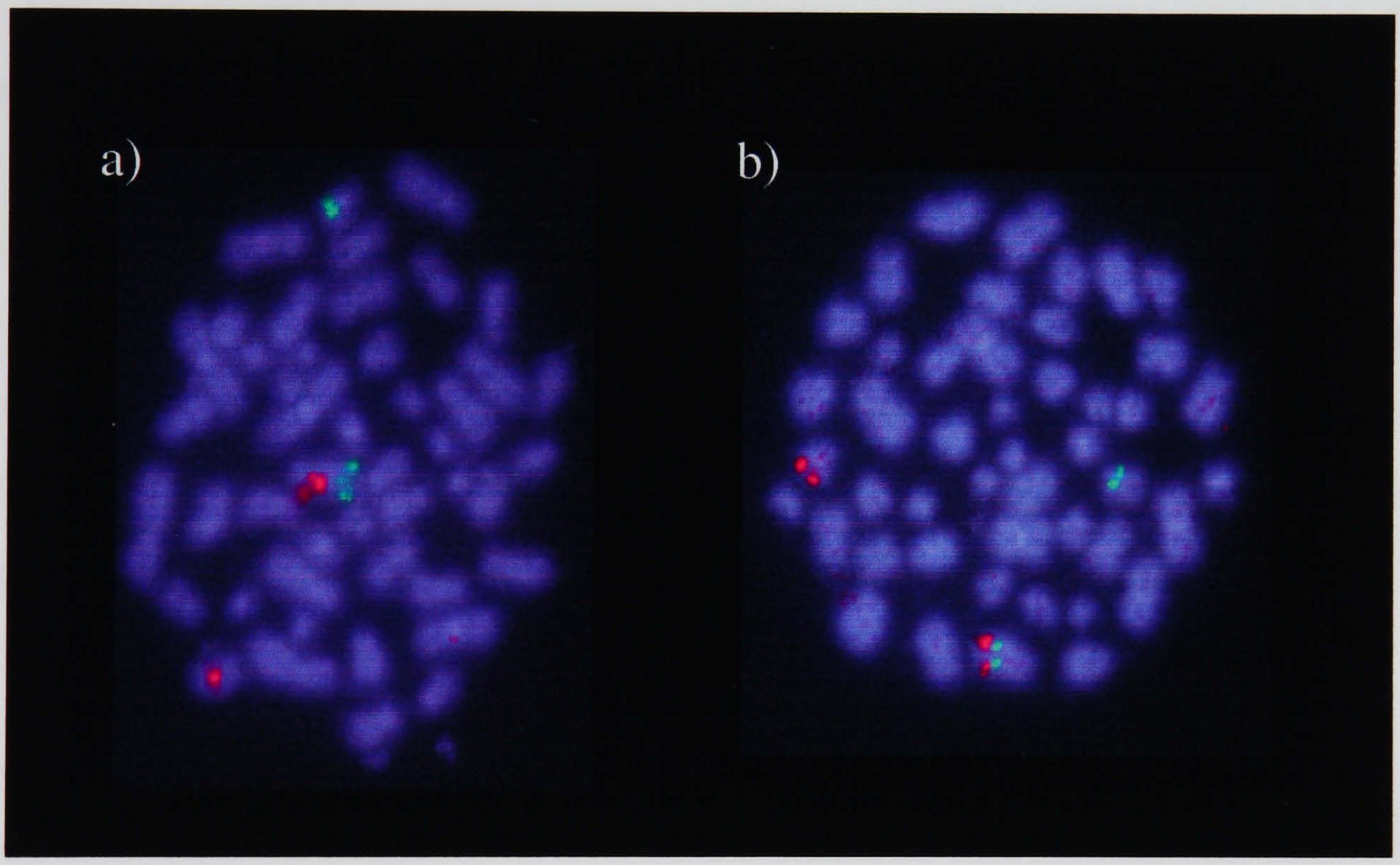


Fig 2.2 Example of FISH probes hybridised to metaphase cells from an ALL patient (Number 29) with a balanced translocation of 6 [in this case t(6;19)]. a) A red 6q centromeric probe marks the normal and derivative 6 chromosomes, the green probe is hybridised to the normal 6 and to the derivative 19 idicating that it is positioned telomeric to the break point in this case. b) A red telomeric 6q probemarks the normal 6 and derivative 19 chromosomes. In this case the green probe is seen on the normal and derivative 6 chromosomes indicating that it is positioned centromeric to the breakpoint.

By performing sequential FISH experiments with probes from the primary panel the boundaries of deletion or the translocation break point was determined in each case. A fresh slide was used for each new set of probes unless all the fixed cells had been used in which case a slide from a previous experiment was prepared for re-FISH as described in **2.5.1**. The HGMP chromosome 6 Database was then searched for additional probes, which could be used to more accurately define deletion boundaries or translocation break points of interest. In some cases the process was repeated several times with the ultimate objective of positioning a break point to within a single clone.

2.6 Gel electrophoresis.

DNA samples to be analysed by gel electrophoresis were mixed with 0.2x volume of loading buffer. For analysis of PCR products and restriction enzyme digests 50ml 0.8-1.2% agarose minigels were made in 0.5x TBE containing 1μg/ml ethidium bromide. Minigels were run in 0.5x TBE as running buffer at 50-100V in Hybaid tanks. For preparation of genomic DNA for Southern blot analysis a 400ml agarose gel was made with 1x AGB and run at 40V overnight using Flowgene apparatus and 1x AGB as a running buffer. Size markers used were bacteriophage λ cut with Hind III, ϕX 174 cut with Hae III (Northumbria Biologicals cat # 030204 and 031201) (Gibco BRL) or a 100 Kb ladder (Hyperladder II, Bioline). In figures (chapter 3) the three markers are denoted by λ Hind III, ϕX and MWM.

2.7 Preparation of Genomic DNA.

DNA was extracted from cell lines using the Purescript DNA isolation kit (Gentra systems). Patient DNA samples had been extracted previously and stored at -20^{0}C by other members of the department.

Cell numbers were measured on a Bayer Advia 120 automated haematology system and approximately 10^7 cells aliquoted into a 15ml centrifuge tube for extraction. Small volumes of lysis buffer were added to the cells until completely lysed. 1 ml of protein precipitation solution was then added to every 3 ml of cell lysate and the mixture vortexed for 20 seconds.

The tubes were then centrifuged at 2,000rpm in a MSE Microcentaur bench top centrifuge for 10 minutes. Supernatant was transferred to fresh centrifuge tubes and a volume of isopropanol, equal to the amount of lysis solution used, was added. Tubes were then inverted approximately 50 times, until a visible DNA precipitate could be seen, then centrifuged at 2,000 rpm for 10 minutes. Supernatant was carefully removed and the pellets transferred to 1.5 ml microcentrifuge tubes and washed with 1 ml of 70% ethanol. After centrifuging at 13,000 rpm for 2 minutes the supernatant was removed and the pellets dried at room temperature. The pellets were then re-hydrated by adding 50µl TE and standing at room temperature overnight. After estimating the concentration by measuring the OD_{260} of a 1:100 dilution, samples were stored at -20^{0}C.

2.8 Preparation of RNA.

RNA was extracted from cell lines using the purescript RNA isolation kit (Gentra systems). Patient RNA samples had been extracted previously and stored at -70^{0}C by other members of the department.

Approximately 10^7 cells were lysed as described in **2.8**, then 1 ml of protein-DNA precipitation solution added to each 3 ml of lysate. Tubes were inverted gently 10 times and incubated on ice for 10 minutes before centrifuging at 2,000 rpm for 10 minutes. Supernatant was transferred to fresh centrifuge tubes, an equal volume (to the lysis solution) of isopropanol was added and the tubes inverted approximately 50 times. The precipitated RNA was then centrifuged at 2,000 rpm for 10 minutes, the supernatant discarded and the pellets transferred to 1.5 ml microcentrifuge tubes. RNA pellets were washed by addition of 1 ml of 70% ethanol then centrifuged at 13,000 rpm for 2 minutes and after discarding the supernatant dried at room temperature. 20-100 µl (depending on pellet size) of PCR grade H_2O was added to the microcentrifuge tubes and the RNA left to rehydrate on ice for 30 minutes. Samples were then vortexed for 5 seconds before storing at -70^{0}C.

2.9 Preparation of first strand cDNA.

1µl of 40U/µl RNasin (Promega cat # N211A) was added to 2µg of RNA in a sterile microfuge tube and the volume adjusted to 20µl with sterile PCR grade H_2O. The reaction was then placed in a 65^0C water bath for 5 minutes then cooled on ice for 5 minutes. The following was then added to the tube in the form of a pre-made master mix; 10µl 5x first strand cDNA buffer (Gibco BRL), 2.5µl 10mM dNTP mix, 1µl RNasin, 1µl 0.5 µg/µl pd(N)6 (Pharmacia), 2µl of Reverse Transcriptase (Gibco BRL cat #), 0.5µl 100mM DTT, 10.5µl sterile PCR grade H_2O.

After mixing the tube was placed in a 37^0C water bath for 1 hour then at 65^0C for 10 minutes and on ice for 5 minutes. The cDNA was diluted with 50µl sterile PCR grade H_20 and stored at -20^0C.

2.10 Evaluation of cDNA using primers from the *G6PD* and *β Actin* genes.

Standard sets of primers for the *Glucose-6-phosphate dehydrogenase* (*G6PD*) and or *β Actin* genes were used in PCR reactions to assess quality of c-DNA. A master mix was made containing the following for each reaction; 2µl 10x buffer (Promega), 2µl 2mM dNTP mix, 2µl 0.1µg/ml forward primer, 2µl 0.1µg/ml reverse primer, 0.2µl Taq DNA polymerase (Promega cat # M186A), 6.8µl PCR grade H_2O.

15µl of the master mix and 5µl of test c-DNA were mixed in a 0.5ml microcentrifuge tube and overlaid with a drop of mineral oil (Sigma cat # M-3516). PCR was performed using a hot start at 94^0C for 10 minutes then 35 cycles at 94^0C for 1 minute, 58^0C for 1 minute and 72^0C for 2 minutes followed by a final extension step at 72^0C for 10 minutes.

5µl of the PCR product was run on a 50ml 1% agarose gel and examined over a UV transilluminator. The *G6PD* primers produced a 168 base pair product and the *β Actin*

primers a 480.base pair product. Quality of test cDNA was determined by evaluating the strength of the band of predicted size produced with the two sets of primers. The sequence of primers for amplification of *G6PD* and *β Actin* are presented in appendix 2.

2.11 Analysis of Gene expression by RT-PCR.

The quality of cDNA was first assessed using primers for the *G6PD* or *β Actin* genes. A PCR reaction was then performed using forward and reverse primers from different exons of the gene to be analysed. The PCR reaction conditions were identical to those described in **2.13** but with the annealing temperature adjusted to 5^0C below the melting temperature of the primers used. PCR products were analysed by running on a 1% agarose gel, and checking for bands of the expected size over a UV transilluminator.

2.12 Mutation analysis of *GluR-6*.

Sequence analysis was used to investigate the possibility that large deletions encompassing the *GluR-6* gene are accompanied by inactivating mutations of the second allele in ALL patients.

2.12.1 PCR amplification of *GluR-6* exons from normal and patient DNA.

Exons of GluR-6 were positioned on sequenced fragments AP002531, AP002530, AP002529, and forward and reverse primers from the flanking genomic regions designed. Primer pairs were designed to amplify the entire exons and splice recognition sites and to have a T_m of either 70 or 65^0C (see appendix 2). Initially, to test the efficiency of the primer pairs, 20µl PCR reactions were set up as described in **2.11** using 100ng of normal (remission) bone marrow genomic DNA. For primer pairs with a T_m of 70^0C the following program was used; 94^0C for 10 minutes then 35 cycles at 94^0C for 1 minute, 65^0C for 1 minute and 72^0C for 7 minutes, followed by a final extension of 72^0C for 7 minutes. For primer pairs with a T_m of 65^0C the annealing temperature was reduced to 60^0C. PCR products were assessed by agarose gel electrophoresis for products of the expected sizes. For amplification of patient DNA, for direct sequencing, 40µl reactions were set up using 200ng of DNA. The PCR

products were assessed by agarose gel electrophoresis and those that resulted in a single intense band of the expected size were purified for sequence analysis. PCR reactions that resulted in either no band or a weak band or more than a single band were repeated. If no satisfactory product resulted from duplication of the original reaction, PCR was performed using 400ng of DNA template. When necessary the additional strategy of reducing the annealing temperature by 2^0C was used to improve efficiency of PCR reactions.

2.12.2 Purification of PCR products for sequencing.

After examining reaction products by gel electrophoresis the remaining 35µl were purified using an Amersham GFX DNA purification kit (cat # 27-9602-01). The procedure makes use of a chaotrophic agent that denatures proteins and promotes binding of double stranded DNA, over 100 base pairs in size, to a glass fibre matrix. Once bound, the DNA is washed to remove contaminating proteins, salts, and primers. Samples were added to 500µl of DNA binding buffer in GFX columns placed in numbered microcentrifuge tubes and centrifuged at 13,000 rpm for 30 seconds. The flow through was discarded and 500µl of wash solution added to each column before centrifuging as before. Columns were transferred to fresh numbered microcentrifuge tubes and 50µl of PCR grade water added to the top of each. After incubating for 1 minute at room temperature the bound DNA was recovered by centrifuging for 1 minute. The eluted PCR products were then diluted with 100µl of PCR grade H_2O and stored at -20^0C.

2.12.3 Direct sequencing of PCR products.

An automated procedure was used to directly sequence the cleaned PCR products. The DNA templates were first labelled by incorporation of four fluorescent dyes (one for each dNTP) using a PCR primer extension technique. Labelled DNA was electrophoresed through a denaturing acrylamide gel the base of which was continuously scanned with a laser beam. As sequence passed through the base of the gel each dNTP was excited by the laser, emitting light of a specific wavelength. The emitted light was collected by a spectrograph and focused onto a cooled charged couple device from where the data was collected and stored by dedicated software. Since each dNTP was labelled with a different fluorochrome, variations

in the wavelength of emitted light could be translated into a genetic code for the electrophoresed DNA molecule.

2.12.3.1 Labeling of PCR products for sequence analysis.

Primers used for the original PCR reaction were prepared at a concentration of 0.8pmoles/µl then separate forward and reverse sequencing reactions containing the following were set up in 250µl PCR tubes; 2µl cleaned PCR product, 2µl primer, 1µl Big Dye (Perkin Elmer), 7µl 2.5x automated sequencing buffer, 8µl PCR grade H_2O. After mixing gently by pipetting the reactions were placed in a Applied Biosystems Gene Amp PCR System 9700 and labelled with the following program; 25 cycles at 96^0C for 10 seconds, 50^0C for 5 seconds and 60^0C for 4 minutes.

Labelled sequences were transferred to 0.5ml microcentrifuge tubes and precipitated by incubating on ice for 1 hour with 2µl 3M Na acetate and 50µl ethanol. The tubes were then centrifuged at 13,000 rpm for 30 minutes, the supernatant discarded and the pellet washed with 70% ethanol. After centrifuging for 10 minutes, the 70% ethanol was removed by pipette and the pelleted DNA dried at room temperature. Labelled DNA was resuspended by vortexing vigorously with 5µl deionised formamide (Amresco, cat # 0606) and 1µl loading dye (Blue Dextran, Perkin Elmer cat # 402055). The resuspended DNA was denatured at 98^0C for 2 minutes then kept on ice until loading.

2.12.3.2 Electrophoresis using the ABI 377 automated sequencer.

All sequencing was performed using a Perkin Elmer ABI Prism 377 sequencing machine with apparatus and protocols supplied by the manufacturer. The plates were washed with sequencing grade H_2O then assembled using 2mm spacers. A gel mix was prepared by dissolving 18g of urea in 25ml sequencing grade H_2O, 5ml of 10x filtered TBE and 5ml of acrylamide/bisacrylamide (40% 19/1) (Kramel Biotech cat # EC-850) solution. The solution was made in the presence of 0.5g of mixed bed ion exchange resin (PE Applied Biosystems cat # 400655) that was then removed by vacuum filtering. The filtered solution was made up to a total volume of 50ml with sequencing grade H_2O. Immediately prior to pouring 35µl of

Temed (Amresco cat # 0761) and 25µl 10% ammonium persulphate were added to the gel. The gel was poured using the supplied gel pouring apparatus and allowed to set for 2 hours.

When set the gel was assembled on the ABI 377 sequencing machine and pre-run with TBE buffer for 20 minutes. 2µl of each denatured DNA sample to be sequenced were then loaded into individual wells and electrophoresis performed for 7 hours.

2.12.4 Analysis of sequenced GluR-6 exons.

Sequences were opened in DNASTAR and edited to remove any nucleotides not lying between or within the forward or reverse primer sequences. The forward and reverse sequences for each exon from each patient were then compared with the complete wild type sequence (including primers and intronic sequence) and the exon sequence alone. If forward or reverse sequence corresponded to the wild type sequence over the entire length of the exon, including the splice recognition sites, the exon was considered not mutated in that patient. If both forward and reverse sequence differed from the wild type sequence at one or more base pair positions, within the exon or at the splice recognition sites, the sequencing reaction was repeated. If after repeating the sequencing, differences between wild type and patient sequence remained at one or more of the same base pair positions, the original PCR reaction was repeated. Repeated PCR reactions were sequenced and compared with both the wild type and earlier patient sequences using the Megalign programme of DNASTAR. If a difference between wild type and patient sequence was found consistently at any one nucleotide position in both forward and reverse directions the exon was considered to harbour either a mutation or polymorphism at that site.

2.13 Southern-blot analysis of IGF2R (patient 22).

2.13.1 Preparation of Hannahan's competent cells.

A single colony of the TG1 strain of *Escherichia coli* was used to inoculate 5ml of SOB medium containing 50µl of 20% glucose and 50µl of 1M $MgCl_2$. After growing overnight at

37^{0}C in a shaking incubator, 500µl of the culture was added to 50ml of SOB medium with 500µl of 20% glucose and 500µl of 1M $MgCl_2$ in a sterile 250 ml flask. The expanded culture was then returned to the incubator and grown to an OD_{600}nm of 0.5-0.55. The flask was then placed on ice for 15 minutes before transferring the cells to a 50 ml Falcon tube. Cells were collected by centrifugation at 1,600 rpm for 10 minutes, the supernatant discarded and the pellet resuspended in 16.5ml of ice cold TFB. After incubating on ice for 10 minutes the cells were centrifuged as before and resuspended in 4ml of TFB. The cell suspension was then incubated on ice with 140µl DnD for 10 minutes before addition of a second 140µl aliquot of DnD. After a further 15 minutes incubation the cells were ready for use and remained competent for 4 hours.

2.13.2 preparation of IGF2-R cloned cDNA.

The complete cDNA sequence of the Insulin Like growth Factor-2 receptor *(IGF2-R)* cloned into the Sal 1 sites of pGEM-2 (known as pGEM-8) was a kindly provided by Dr S. Sly [Department of Biochemistry and Molecular Biology, St Louis University, USA]. The lyophilised DNA was resuspended in TE at 50 ng/µl and 1 and 5µl aliquots added to 200µl competent TG1 cells on ice for 45 minutes (see **2.13.1**). Following incubation on ice the cells were heat shocked by placing in a 42^{0}C water bath for 2 minutes then incubated with 400µl 2xTY at 37^{0}C for 30 minutes before plating onto LB or TY agar containing ampicillin at 100µg/ml. Individual colonies were then used to inoculate 50ml LB medium containing ampicillin and DNA prepared by standard maxi-prep (see 2.4.1).

2.13.3 Construction of probes for Southern Blot analysis.

PGEM-8 DNA was then used as a template for the construction of 5 probes, covering the 48 exons of the IGF2-R, by PCR. The forward and reverse primer sequences used to amplify each probe, their base pair positions on the IGF2R and the exons covered by each set of primers are shown in Appendix 2. 50µl PCR reactions containing: 0.5µl Promega Taq polymerase (cat # M186A1), 5µl Promega Taq buffer, 5µl 2mM dNTPs, 5µl each primer (100ng/µl) and 100ng of pGEM-8 DNA were set up for each pair of primers. After covering with a drop of mineral oil the PCR reactions were denatured at 94°C for 10 minutes then

cycled 25 times at 94°C for 1 minute, 65°C for 1 minute and 72°C for 4 minutes followed by a final extension at 72°C for 10 minutes using a Techne Cyclogene thermal cycler (PH3). Satisfactory PCR products were obtained from primer sets 2-5 but not from set 1. PCR reactions were therefore carried out using the same conditions but using a forward primer from the SP6 promoter region of pGEM-2 (see Table 2.1 for sequence) with reverse primers from sets 1 and 2. A suitable PCR product resulted from the combination of SP6 forward and the reverse primer from set 1, the probe derived from this PCR product will be referred to as IGF2R SB probe 1. The probes derived from the products obtained from primer sets 2–5 will be termed IGF2R SB probes 2-5. The IGF2R SB probes were purified by running the PCR products on a 1% agarose gel, and excising the bands of expected size with a scalpel blade over UV light. The excised agarose fragments were placed on top of small pads of glass wool in 0.5ml microcentrifuge tubes. The bottoms of the tubes were punctured with a pin and then placed inside 1.5ml microcentrifuge tubes and centrifuged at 13,000 rpm for 1 minute. The extruded DNA solutions were cleaned once with PCIA and once with chlorophorm/isoamyl-alcohol (24:1;v:v), then passed through a Sephadex G50 column. To estimate the DNA concentration 5µl was run on a 50ml 1% agarose gel and band intensities compared with those of λ HindIII marker bands of known concentration.

2.13.4 Restriction Enzyme Digest of Genomic DNA.

Stored DNA from bone marrow samples from patient 22 taken at 2 different dates (between 13.02.98 and 21.09.98) and from three lymphoma patients (identification numbers B22A6, B22A7 and B22A8) were used for Southern Blot analysis. Master mixes for EcoR1 and BamH1 digestion of DNA containing the following per reaction were prepared; 5µl restriction enzyme digestion buffer, 0.5µl spermidine, 0.5µl DTT, 4 µl enzyme (EcoR1 or BamH1) (New England Biolabs / 20U/µl activity), 30µl H_20. Each digest consisted of 10µl of one of each of the 5 DNA samples (1µg/µl) and 40 µl of master mix. The 10 digests were incubated in a water bath at 37^0C for 16 hours then loaded onto a 0.8% agarose gel made with 1 x AGB buffer and run at 40V overnight.

2.13.5 Southern Blot Procedure.

After electrophoresis the gel was examined over UV light, an image taken and the position of size marker bands indicated with ink using a 19-gauge needle. The gel was then placed in depurination solution and gently agitated for 20 minutes. Depurination of sites within DNA allows cleavage during denaturation resulting in fragmentation of longer pieces of DNA and allowing efficient transfer of high molecular weight DNA to the membrane. The gel was then rinsed once in distilled H_2O and soaked in denaturing solution for 30 minutes. After rinsing once more in distilled H_2O the gel was transferred to neutralising solution for 30 minutes.

While preparing the gel, 1L of 20xSSC was added to a transfer tank containing a platform overlaid with 2 sheets of Whatman 3MM paper. The ends of the Whatman paper were submerged, acting as wicks to draw up the 20xSSC. The prepared gel was then carefully placed on the saturated Whatman paper and overlaid with Saran wrap. The Saran wrap was cut away from around the edge of the gel and a piece of pre-wetted Hybond-N and 4 pieces of pre-wetted Whatman 3MM were then laid over the gel. 5-10 cm of paper towels were stacked over the Whatman paper and weighted with a suitable heavy object. The Southern blot apparatus was then left overnight to allow transfer of DNA to the Hybond membrane by capillary action.

The weight and papers were removed from the blot and the gel and Hybond membrane inverted onto a glass plate. The position of molecular weight marker bands and loading wells were marked on the membrane before discarding the gel. The membrane was then washed twice for 15 minutes in 3xSSC, baked in an oven at 80^0C for 2 hours and cross-linked with UV light (using a Stratalinker). Immediately prior to hybridisation the membrane was soaked in 3x SSC for 30 minutes at room temperature, then pre-hybridised in 20ml Church buffer at 65^0C for 1-2 hours. Prehybridisation and hybridisation were performed using a Hybaid hybridisation apparatus.

2.13.6 Probe labelling and hybridisation.

50 ng of IGF2R SB probe 1 was made up to a total volume of 50µl with PCR grade H_2O placed in a boiling water bath for 5 minutes then transferred to ice for 5 minutes. The denatured probe was added to a pre-prepared random prime labelling mix (Rediprime, Amersham life sciences) with 2µl of [α-^{32}P]dCTP (10µCi/µl) and incubated at 37^{0}C for 1 hour. Labelled probe was then made up to a total volume of 100µl with PCR grade H_2O and unincorporated nucleotides separated from the probe by centrifuging through a Sephadex G50 column. The Sephadex column was prepared in a 1ml-syringe barrel plugged with glass wool and equilibrated with distilled H_2O. After cleaning, the labelled probe was denatured by boiling for 5 minutes and immediately added to the pre-hybridising membrane. Hybridisation was carried out overnight at 65^{0}C. To remove non-specifically bound probe, the membrane was washed 3 times in 0.5x SSC/0.2% SDS in a shaking water bath for 20 minutes at 65^{0}C. A storage phosphor screen (Packard) was exposed to the washed membrane for 72 hours and imaged on a Packard Cyclone storage phosphor system.

After imaging, IGF2R SB probe 1 was stripped from the membrane by washing for 2 minutes in denaturing solution at room temperature on a rocking platform and for 2 minutes in neutralising solution at room temperature. IGF2R genomic probes 2-5 were labelled and hybridised to the membrane sequentially, with probe from the previous experiment being stripped before each fresh hybridisation.

2.14 RACE-PCR analysis of IGF2R (patient 22).

2.14.1 Principle of the SMART RACE PCR system.

Rapid amplification of cDNA ends (RACE) PCR techniques have been developed for the purpose of identifying unknown exons spliced to either the 5' or 3' end of an already characterized expressed sequence[262]. The full-length transcripts of many genes have been cloned in this way but the technique has also been used to identify partner genes of oncogenic translocations where one partner is known. The SMART RACE system (Clontech cat # K1811-1) involves two steps. 1) The preparation of cDNA templates appropriate for the

amplification of 5' (5' RACE ready cDNA) and 3'(3' RACE ready cDNA) ends of genes. 2) The amplification of 5' or 3' sequences from the cDNA templates using gene specific primers.

cDNA is prepared using a mutant form of MMLV reverse transcriptase which adds an overhanging CCC sequence after it reaches the end of an RNA template (Clontech PowerScript Reverse Transcriptase cat # 84601). The 5' RACE ready reaction contains 2 primers one of which is designed to hybridise to 3' poly A tails (5'- CDS) and a second (the SMART II oligonucleotide) consisting of a universal primer tagged to a GGG sequence which hybridises to the CCC overhang added by the mutant MMLV Reverse Transcriptase. Reverse transcription proceeds from the 3' poly A tail to the 5' end of the gene where the SMART II oligonucleotide anneals to the added CCC sequence. At this point Reverse Transcriptase switches templates adding the complement of the universal primer to the 1st strand cDNA sequences. The 5' end of genes can be amplified from the cDNA using the universal primer sequence as a forward primer in combination with gene specific reverse primers.

The 3' RACE ready reaction contains a 3' cDNA synthesis primer (3'-CDS) consisting of an oligo-dT, which hybridises to 3' poly A tails, coupled to the universal primer. Reverse transcription advances from the poly A tail towards the 5' end of the gene resulting in production of cDNA templates with a sense version of the universal primer attached to the 3' end. Using a combination of gene specific forward primers and the universal primer (as reverse primer) the 3' ends of genes can be amplified by PCR from the 3' RACE ready cDNA.

A nested PCR reaction can also be achieved by performing a first round PCR with a universal primer mix and a touchdown program. The universal primer mix contains two different primers, a long universal primer consisting of the universal primer sequence with an additional 22 base pair sequence on its 5' end and a short universal primer consisting of the 22 base pair sequence alone. The first few cycles of PCR are carried out using a relatively high annealing temperature at which the long universal primer can pair with the template

cDNA adding the 22 base pair short primer sequence to the original cDNA template. Further cycles are carried out at a reduced temperature allowing the short primer to amplify the extended template. First round PCR products made using this protocol can then be used as a substrate for nested PCR using the universal primer sequence (nested universal primer) in combination with nested gene specific primers.

2.14.2 Strategy for cloning a putative unknown fusion partner of the IGF2-R.

A translocation of chromosome 6 observed in patient 22, was shown by FISH to disrupt the *IGF2-R* (see **3.6.4**), with a break point estimated to fall between exons 4 and 5 of the gene. To investigate the possibility that the translocation resulted in production of a fusion gene, 5' and 3' RACE PCR were performed using the SMART RACE system. Forward primers from exons 1-3 were designed for the 3' RACE reactions and reverse primers from exons 7-10 for the purpose of cloning the 5' end of any *IGF2-R* fusion gene. Products resulting from nested RACE PCR reactions were cloned into plasmid vectors and sequenced. BLAST search was then carried out on any non-*IGF2-R* sequence. An overview of the approach is shown in figs **2.3** and **2.4.**

2.14.3 Evaluation of patient RNA.

Stored RNA made from a bone marrow sample was taken from patient 22 on 21.09.98 (when the chromosome 6 translocation was present in approximately 50% of cells) and used for RACE-PCR analysis. The patient RNA sample was first checked for quality and quantity by running on a 50 ml 1% agarose gel with a commercially prepared placental RNA sample (Clontech). The following precautions were taken to prevent RNA degradation: water containing 0.1% (DEPC) was used for the preparation of the gel. The gel former and tank were washed with detergent, dried with ethanol then soaked in 3% hydrogen peroxide for 10 minutes before a final wash with the DEPC water. 0.5 x TBE was made with the DEPC water and used both to make a 1% agarose gel and to run the gel after loading. 3µl each of the placental RNA (1µg/µl), the patient RNA and a 1:10 dilution of the patient RNA were run on the gel.

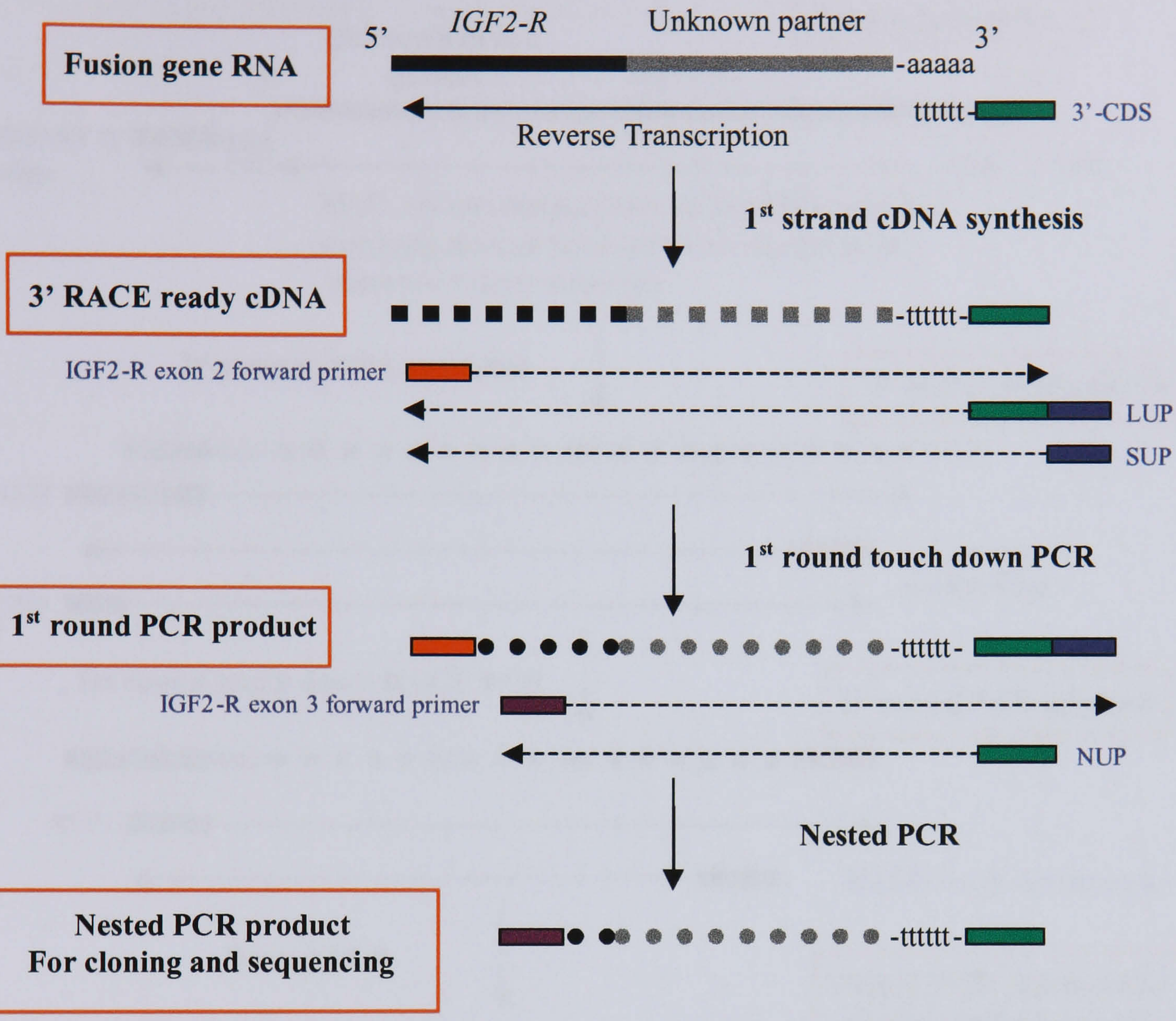


Fig 2.3 Strategy for cloning the 3'end of the putative unknown partner gene of the *IGF2-R*. 3'CDS (3' RACE cDNA Synthesis Primer), LUP (Long universal primer), SUP (Short universal primer), NUP (Nested universal primer).

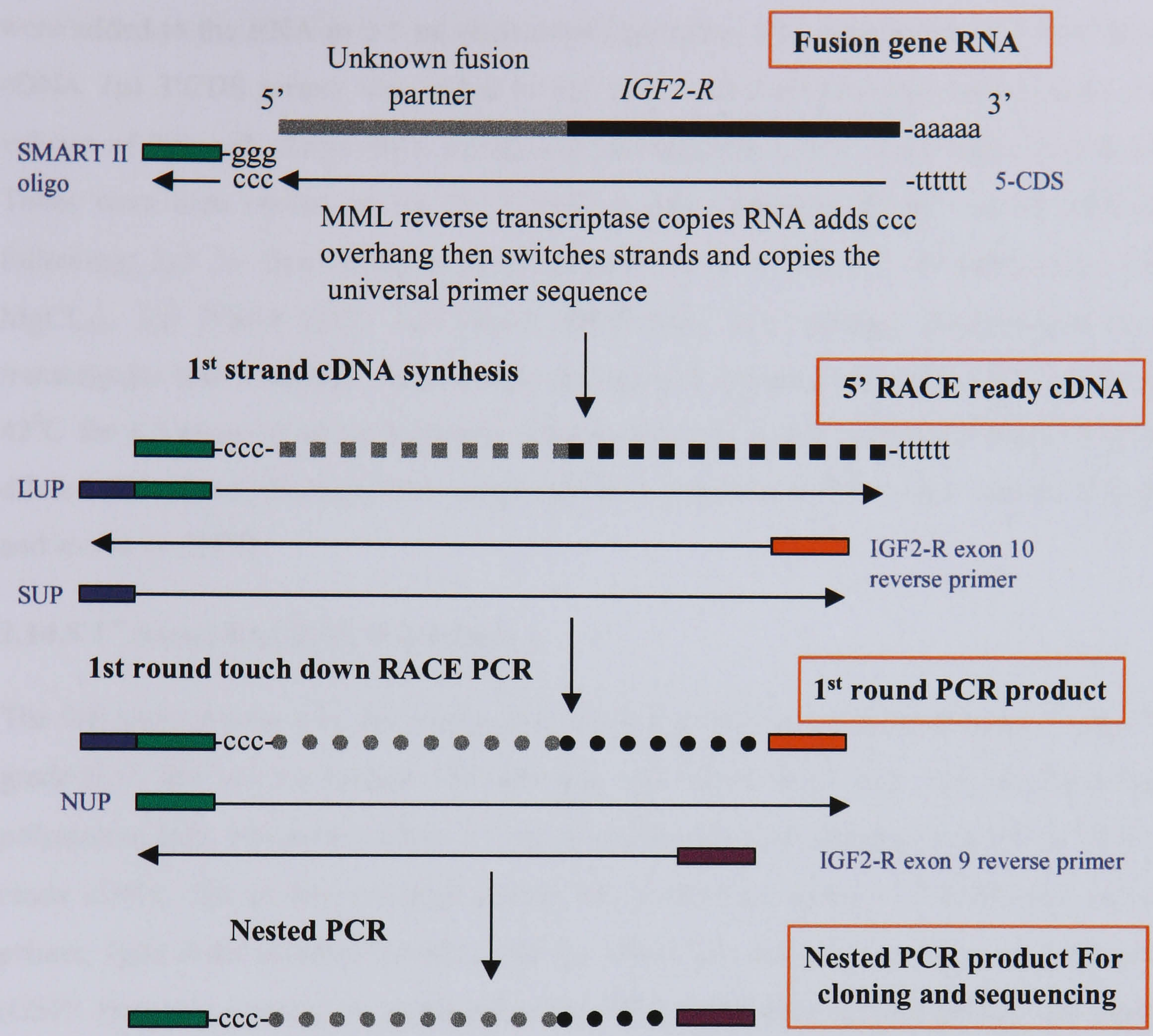


Fig 2.4 Strategy for cloning the 5' end of the putative fusion partner of the *IGF2-R*. 5'CDS (5'-RACE cDNA Synthesis primer), LUP (Long universal primer), SUP (Short universal primer), NUP (Nested universal primer)

2.14.4 Preparation of RACE ready cDNA.

5' and 3' RACE ready cDNA was prepared from the patient RNA and supplied positive control placental RNA using a SMART RACE kit (Clontech cat # K1811-1) following the manufacturers protocol (oligonucleotide sequences for these reactions are presented in appendix 2). 1µl (1µg) positive control RNA or 3µl patient RNA were used for each reaction. For the preparation of 5' RACE ready cDNA 1µl 5'CDS primer and 1µl SMART II oligo

were added to the RNA in 0.5 ml microcentrifuge tubes. For preparation of 3' RACE ready cDNA 1µl 3'CDS primer was added to the RNA. All reactions were made up to a total volume of 5µl with sterile H_2O, mixed and incubated in a 70^0C water bath for 2 minutes. Tubes were then cooled on ice for 2 minutes and centrifuged briefly before adding the following; 2µl 5x first strand buffer (250mM Tris-HCL pH 8.3, 375mM KCL, 30mM $MgCL_2$), 1µl 20mM DTT, 1µl 10mM dNTP mix, 1µl Clontech PowerScript Reverse transcriptase (cat # 84601). After briefly mixing and spinning, the tubes were incubated at 42^0C for 1.5 hours in an air incubator. The resultant 1st strand reaction products were then diluted with 100µl Tricine-EDTA buffer (pH 8.5), placed in a 72^0C water bath for 7 minutes and stored at -20^0C.

2.14.5 1st round RACE PCR reactions.

The following master mix was prepared (volumes given for a single reaction). 34.5µl PCR grade H_2O, 5µl 10x Advantage 2 PCR buffer, 1µl 10mM dNTP mix, 1µl 50x Advantage 2 polymerase mix. For each reaction 41.5µl of master mix was added to 2.5µl 3' or 5' RACE ready cDNA, 5µl of 10x universal primer mix (UPM consisting of 0.4µM long universal primer, 2µM short universal primer), and 1µl 10µM forward or reverse gene specific primer (GSP). Negative controls in which either the UPM or the gene specific primer was replaced with an equal volume of PCR grade water were included. For positive control reactions GSPs for the transferin receptor (TFR), supplied with the SMART RACE II kit, were used. The PCR reactions were performed on an Applied Biosystems Gene Amp PCR System 9700 using the following program for the first round PCR.

5 cycles	94°C 5 sec, 72°C 3 min
5 cycles	94°C 5 sec, 70°C 10 sec, 72°C 3 min
25 cycles	94°C 5 sec, 68°C 10 sec, 72°C 3 min

All primers used for 1st round and nested PCR reactions (see section 2.14.6) are presented in appendix 2 . PCR products were assessed by running 5µl on a 1.2% agarose gel, remaining product was stored at -20^0C.

2.14.6 Nested RACE PCR reactions.

For nested PCR reactions 5µl 1st round PCR products were diluted in 245µl tricine-EDTA buffer. 5µl of the diluted product was then added to 41.5µl of the master mix described in 2.9.5, with 1µl nested universal primer, 1µl nested GSP and 1.5µl PCR grade H_2O. The following program was used for nested PCR reactions;

25 cycles	94°C 5 sec, 68°C 10 sec, 72°C 3 min
1 cycle	72°C 7 min.

Nested PCR products were assessed by running 5µl on 50ml 1.2% agarose gels.

2.15 Cloning of SMART RACE PCR products.

SMART RACE PCR products were cloned using a TOPO TA cloning kit (Invitrogen cat # 45-0641). The TOPO cloning system depends on the non-template dependent terminal transferase activity of Taq polymerase that results in addition of a single deoxyadenosine to the 3' end of PCR products. The supplied linearised vector (pCR 2.1-TOPO) has single overhanging 3' deoxythymidine residues which allow efficient ligation of PCR inserts. The activated form of the vector has topoisomerase I molecules covalently bound to the 3' phosphate of the thymidine residues. The covalent bond to topoisomerase I is broken and the released energy used to complete a DNA phosphodiester backbone in the presence of DNA 5' hydroxyl groups. Introduction of PCR products to the vector provides both a template for the single base pair overhangs and 5' hydroxyl groups that cause release of topoisomerase and provides energy for the ligation of insert to vector.

2.15.1 Cloning procedure.

Cloning reactions containing the following were set up in 150µl PCR tubes; 1-3µl fresh PCR product, 1µl salt solution (1.2 mM NaCl, 0.06 M $MgCl_2$), PCR grade H_2O to a total volume of 6µl, activated TOPO vector 1µl. The reactions were mixed gently incubated at room temperature for 5-30 minutes then placed on ice.

2µl of cloning reaction was then added to 200µl competent cells (either thawed frozen cells supplied with the kit or freshly prepared TG1 cells) mixed gently and placed on ice for 5-30 minutes. The cells were then heat shocked by placing in a water bath at 42^{0}C for 30 seconds and immediately transferred to ice. 400µl 2xTY was added to each tube before incubation for 1 hour at 37^{0}C in a shaking incubator. 200µl and 400µl aliquots from each culture, with 40µl 40mg/ml X-Gal, were then spread on pre-warmed LB plates containing 50µg/ml kanamycin. Plates were incubated overnight at 37^{0}C.

2.15.2 Selection of clones for sequencing.

After overnight incubation white but not dark blue, colonies were selected for further analysis. To confirm the presence and determine the size of cloned DNA sequences a rapid PCR based assay was performed on individual colonies. Using a sterile tooth pick cells from each colony were first smeared onto the bottom of a numbered PCR tube then streaked onto a single square of a gridded agar plate. A number corresponding to one of the PCR tubes identified each square of the grid so positive colonies could later be identified and sequenced. The agar plates were incubated at 37^{0}C overnight then stored at 4^{0}C. To amplify insert sequences 10µl PCR reactions were set up in each of the numbered tubes. A master mix containing the following (per reaction) was prepared; 1µl 10x buffer (PerkinElmer), 1µl 2mM dNTP mix, 1µl nested universal primer, 1µl GSP (as used to produce the cloned product), 0.1µl Taq polymerase (PerkinElmer), 5.9µl PCR grade H_2O. After addition of 10µl master mix tubes were place in a an Applied Biosystems Gene Amp PCR System 9700 and cycled 25 times at 94^{0}C for 30 seconds, 68^{0}C for 30 seconds and 72^{0}C for 3 minutes. PCR products were sized by running on a 1% agarose gel. Clones containing inserts similar in size to the bands obtained by RACE PCR were then picked from the gridded agar plates and used to establish small-scale cultures for DNA preparation and sequencing.

2.15.3 Small-scale DNA preparation of RACE PCR clones (miniprep).

2 ml 2xTY cultures were inoculated with the selected clones and grown overnight at 37^{0}C. DNA was then purified using a Sigma GenElute plasmid miniprep kit (cat # PLN 350). Following a modified alkaline-SDS lysis procedure plasmid DNA was adsorbed onto a silica

membrane in the presence of high salts. Contaminants were removed by spin-wash and the DNA eluted with H_2O.

1.5 ml of culture was transferred to a microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute and. After discarding the supernatant the pellet was resuspended in 200µl of resuspension solution (containing RNase A) by vigorous vortexing. 200µl of lysis solution was then added and the cell suspension mixed by inverting 6-8 times to produce a clear viscous solution. Cell debris, proteins, lipids, SDS and chromosomal DNA were precipitated, by adding 350µl of neutralization solution and gently inverting the tube 3-4 times. The precipitate was pelleted by centrifugation at 13,000 rpm for 10 minutes and the supernatant loaded onto a binding column. The column, resting in a microcentrifuge tube, was then centrifuged at 13,000 rpm for 30 seconds and the flow through discarded. The bound plasmid DNA was then washed by adding 750µl of wash solution to the column and centrifuging at 13,000 rpm for 30 seconds. After discarding the flow through excess ethanol was removed from the column by centrifuging for 2 minutes. The columns were transferred to fresh microcentrifuge tubes and DNA eluted by adding 50µl PCR grade H_2O and centrifuging for 1 minute at 13,000 rpm. Recovered DNA solutions were stored at -20^{0}C.

2.15.4 Restriction enzyme digest of cloned DNA.

The insertion sites of the pCR 2.1-TOPO vector are flanked by EcoRI restriction enzyme sites. To confirm the presence of insert sequences in clones, before sequencing, a restriction enzyme digest was carried out. Digests were carried out at 37^{0}C for 1.5 hours and consisted of 5µl of clone DNA, 3µl of restriction digestion buffer, 0.3µl DTT, 0.3µl spermidine, 1.0µl EcoRI and 20.4µl PCR grade H_2O. 10µl of each digest was then run on a 50 ml 1% agarose gel and clones containing inserts selected for sequencing.

2.16 Sequence analysis of cloned DNA.

Sequencing was carried out essentially as described in **2.13.4** using 2µl of purified cloned DNA and 1µl of forward or reverse primer, supplied with the kit and hybridizing to vector sequences flanking the insert site.

Before analyzing sequences for evidence of the presence of an *IGF2R* fusion partner gene, vector sequences were removed by editing the sequence using the DNASTAR software. The trimmed sequence was then compared with the wild type *IGF2R* sequence. To determine the origin of non-*IGF2R* sequence, BLAST search was conducted using the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Chapter 3 (Results)

3.1 Karyotypes (G-banded analysis).

The full reported karyotypes of patients and cell lines for which successful FISH analysis was carried out are presented in **Table 3.1**. The analysed cases include 21 ALL patients reported to carry a cytogenetically detectable deletion of 6q, two translocations of 6q (cases 26 and 29) and one case with monosomy 6 and markers of unknown origin (case 23).

Of the 7 patients with AML, 2 had deletions, one had an unbalanced translocation and 5 had balanced rearrangements of 6q. One of the AML patients (case 32) was reported to carry a deletion as well as a translocation and an inversion of chromosome 6.

Of the 3 cell lines (cases 5, 13 and 16) all were derived from T-ALL patients and had deletions of 6q that were confirmed by G-banded analysis.

Patient or Cell line	Karyotype (G-banded analysis)
1	46,XX,del(6)(q?13q21),add(9)(p24),del(13)(q?14),der(19)t(1;19)(q23;p13)
2	46,XX,del(6)(q15q23),i(9)(q10),der(19)t(1;19)(q23;p13)
3	46,XY,del(6)(q21)

4	46,XY,**del(6)(q21q25)**,del(9)(p13)
5	79-91<4n>,XXX,-X,der(1)t(1;9)(p35~36q11),+3,+3,dup(4)(q13q23)x2, **del(6)(q14q22)x2**,-10,t(11;14)(p15;q11)x2,-13,add(13)(q34),-14,+15, add(15)(p13),-18,-20,+2mar[cp]
6	46,XY,**del(6)(q1?q21)**
7	46,XY,**del(6)(q13q23)**
8	46,XY,**del(6)(q21**),t(8;14)(q24;q11)
9	46,XY,**del(6)(q?16q?24)**
10	47,XY,der(2)add(2)(p)del(2)(q),**del(6)(q21q23)**,add(9)(p13), add(11)(q23),+21
11	46,XY,**del(6)(q?)**,del(12)(p?)
12	46,XY,**del(6)(q2?q2?)**
13	89-99<4n>,XXYY,+4,**del(6)(q16)x2**,+7,der(7)t(7;7)(p15;q11)x2, +8,+20,+20[cp]
14	46,XY,**del(6)(q?15q2?3)**
15	46,XY,**del(6)(q13q21)**
16	42-47,XX,der(4)?dup ins(4;4)(p?11;q?21q25),del(5)(q22q31), **del(6)(q13q22)**,del(9)(p11p22),del(9)(q22)[cp]
17	46,XX,**del(6)(q21q21)**

18	45,XY,t(2;3)(p15;q26),del(6)(q13~15q21),add(9)(p11~13),del(9)(q32q34), der(12)t(12;17)(p11;q11),-17[26]/45,idem,del(3)(p?23p25)[4]
19	58,XY,+X,t(2;11)(p1?6;q13),+4,+5,+6,del(6)(q22),+10,+13,+14,+16, +17,+18,+20,+21,
20	45,XX,dup(5)(q22q31),del(6)(q21q23),-7,der(9)t(7;9)(q11.2;p24), del(14)(q13q24),-16,-18,der(22)t(?;22),+mar1,+mar2[13] /45,idem,del(10)(q22q25)[3]/44,idem,-2,del(10)(q22q25)[4]
21	44,XY,-3,del(4)(q ?),del(6)(q21),-7,der(10)t(?;10)(?;p11.2),+i(10)(p10), -11,-12,der(12)t(3;12)(p13;p11),+?i(12)(p10),del(15)(q ?),-17, der(17)t(?;17)(?;p11.2),-18
22	47,XY,add(2)(q2?),?inv(3)(p2?4q21),?del(6)(q2?1),add(7)(q11), -8,t(8;15)(p?11;q1?),add(12)(p1?),add(13)(q3?4),der(16)t(7;16)(q11;p13), add(17)(p13),der(20),add(20)(q1?),-21,+3mar
23	46,XX,t(1;19)(q23;p13),-6,add(8)(q24),+mar
24	46,XX,t(9;22)(q34;q11)[6]/46,idem,der(6)t(1;6)(q?23;q?21)[11] /49,idem+8,+19,+der(22)t(9;22)(q34;q11)[2]/50,idem,+8,+19,+22, +der(22)t(9;22)(q34;q11)
25	45,XY,del(6)(q21),i(9)(q10),der(12)t(12;17)(p11;p11),-17
26	51,XY,+X,t(2;18)(p13;q23),?t(3;10)(q21;q26),t(3;16)(p25;p13), t(6;9)(q2?5;q22),inv(12)(p12q21),+14,del(17)(q21;q21),+20,+21,+mar,
27	46,XY,del(6)(q?13q?21),add(7)(q3?),del(9)(p1?3),t(11;14)(p13;q11)
28	46,XY,del(6)(q?),del(10)(q?),add(20)(p?)

29	46,XX,der(18;21)(q10;q10)[4]/87<4n>,XX,-X,-X,-1,-3,add(4)(q?31)x2, der(5)t(5;21;15)(q22;q22;q1?5)t(12;15)(p13;q22)x2,-6,-6, t(6;19)(q?21;p13)x2,t(7;22)(q22;q13),+der(7)t(7;22)(q22;q13), del(7)(q22q36)x2,t(8;11)(p?10;q1?4),del(11)(q1?4q23)x2,-16, +der(19)t(6;19)(q?21;p13),der(21)t(6;21)(p?21;q22)x2[5]
30	46,XX,t(6;11)(q13;q23)
31	46,X,?t(X;22)(q2?4;q11),?t(4;13)(q2?5;q1?),?t(6;7) (q2?1;p15),del(15)(q11q?21)
32	48,XY,+6,del(6)(q?21q2?5),inv(6)(p13q22),der(7)t(7;17)(p10;q10), t(7;8)(p1?3;q1?1),der(15)t(15;17)(q2?5;q10),+22[3]/48,idem, -del(6)(q?21q2?5),t(8;9)(q22;q32),+mar[5] /47,idem,t(2;6)(q3?1;q23),?inv(5)(q1?q22),?der(6)t(6;6)(q2?21;p10), -del(6)(q2?21q2?5)[4]
33	46,XX,add(6)(q23)
34	46,XY,t(6;8)(q27;p11.2)

Table 3.1 Karyotypes of patients/cell lines included in the study. 6q abnormalities are highlighted in red.

3.2 Primary FISH analysis.

PACs or BACs, used for initial FISH analysis, were selected through the ACEDB database on the basis of their genetic marker content. The markers used for clone selection were either taken from publications in which they had been used for LOH analysis of cases of ALL, or were positioned on the YACs used to map deletions in our own department[256]. Additional clones containing genes either known or thought to be involved in malignancy were also selected for initial analysis. To achieve a more comprehensive coverage of 6q further PACs,

assigned to a cytogenetic band by FISH, were included in this primary panel of probes (personal communication from A. Mungal Sanger Centre, Hixton Cambridge UK). The probes used with cytogenetic band assignments, corresponding genetic markers and genes and chromosome 6-fpc values are presented in **Table 3.2**. Of the 34 cases analysed, 24 were found to bear deletions and 12 translocations of 6q (patients 22 and 23 were associated both with translocation and deletion).

PAC P13743 (marked with * in **Table 3.2**) was not part of the panel of clones used by the Sanger center in map construction and therefore not assigned a 6-fpc map position by ACEDB. However, P13743 was known to contain the *CCNC* gene[287] that on earlier releases of the chromosome 6 physical map was positioned between RP1-235B9 and RP1-202B13. Three colour FISH on interphase cells placed P13743 between RP1-202B13 and YAC 748c8 that contains the marker D6S283 (**Fig 3.1**)[256]. The marker D6S283 had been positioned telomeric to RP1-202B13 so interphase FISH placed *CCNC* between RP1-202B13 and RP1-132E6. At the time of initial analysis the assumption was made that the HGMP database order:centromere-RP1-235B9, P13743 (*CCNC*), RP1-202B13-telomere was correct and the three probes were placed accordingly in **Tables 3.2, 3.3** and **3.4** and in **Fig 3.2** and **3.3**. A later release of the chromosome 6 map was in agreement with the order derived from interphase FISH analysis, placing CCNC distal to RP1-202B13 (see **3.4.2**).

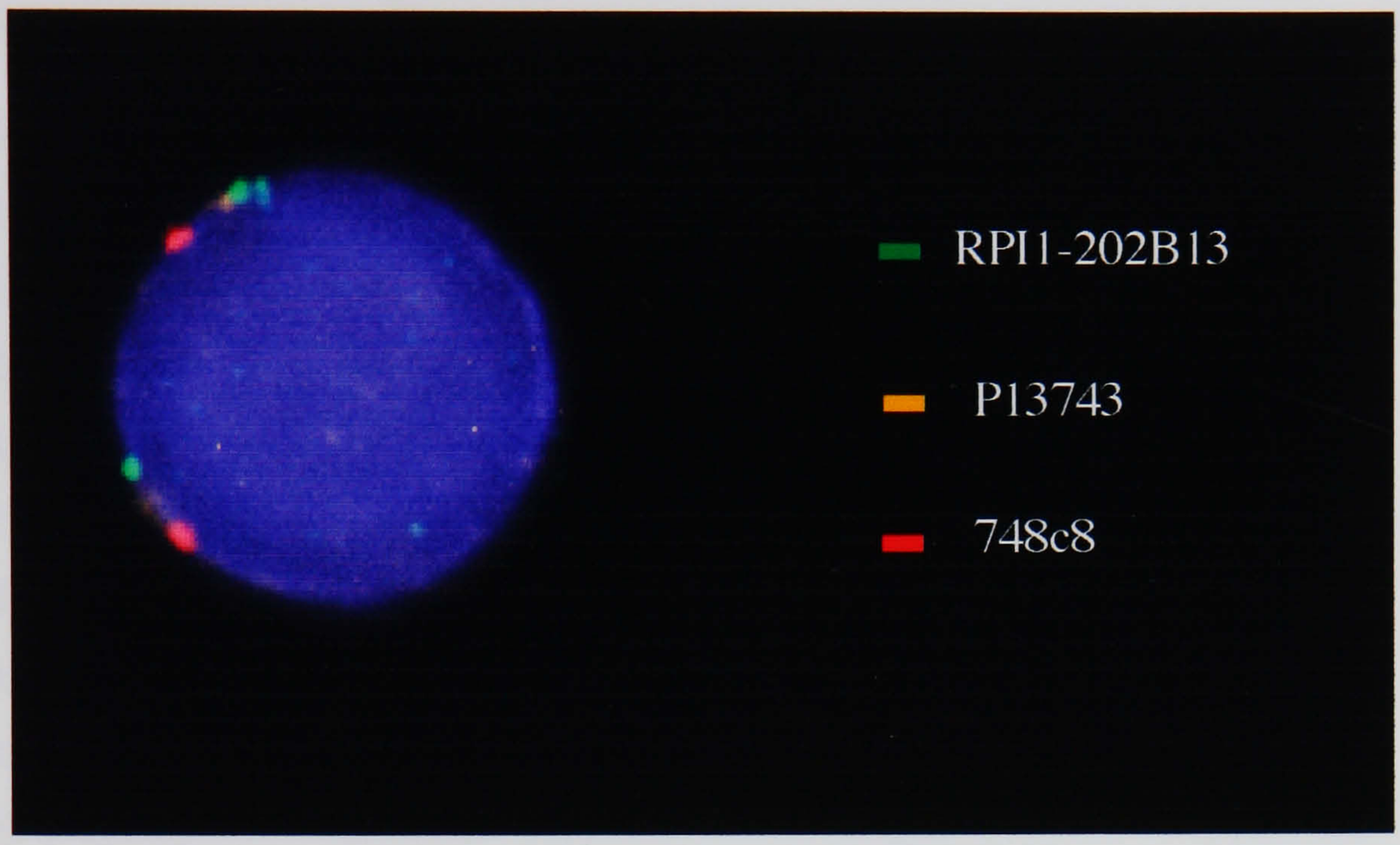


Fig 3.1 PAC P13743 (containing the gene *CCNC*) is positioned to between PAC RPI1-202B13 andYAC 748c8 (containing marker D6S-283) using 3 colour FISH on interphase cells. RPI1-202B13 was labelled with Spectrum Green, P13743 with Alexia 532 and 748c8 with Spectrum Red.

Probe	Chromosome 6 fpc values	Cytogenetic band	Marker	Gene
RP1-71H19	59950-60050	q11		PRIM2A
RP1-260B21	79950-80050	q14	D6S1625	
RP1-102H19	90250-90400	q15	D6S1004	
RP1-67N15	91650-91750	q15	D6S1185	
RP1-235B9	93550-93650	q15	D6S462	BACH2
P13743*		q16		CCNC
RP1-202B13	100600-100700	q16	D6S501	
RP1-132E6	108150-108300		D6S447	
RP11-59I9	110200-110300	q21		
RP1-429G5	111150-111200	q21	D6S1594	
RP1-111B22	111100-111150	q21	D6S1113	
RP1-66H14	114450-114600	q21-22	D6S302	FYN
RP1-240M13	116600-116750	q22	D6S499	
RP1-162C6	117950-118050	q22		
RP1-59B14	121250-121400	q22	D6S433	ERV9
RP1-293L8	128350-128500	q22	D6S1311	HEY2
RP1-179E13	129200-129350	q22	D6S1076	
RP1-76H23	135800-135900			
RP1-171N11	141100-141300	q23		
RP1-172K10	142850-143000	q24		
RP1-178D18	144050-144200		D6S1648	
RP1-237G20	149550-149650	q25		
RP1-278N12	156950-157100	q25	D6S425	
RP1-137K2	159450-159650	q25	D6S1878	
RP1-65A9	170802-170937		D6S5481	
RP1-167A14	178304-178414	q27		FOP
RP1-57M24		q27		6q telomere

Table 3.2 Probes used in the primary FISH analysis of patients and cell lines, showing associated HGMP chromosome 6 map positions, chromosomal band assignments, genetic markers and genes.

3.2.1 Patients and cell lines with deletions.

Numbers of metaphase cells deleted for or retaining each probe, in each case, are recorded in **Table 3.3**. 24 patients/cell lines had deletions that included a region corresponding to one or more of the probes used in the primary analysis. For each case, probes that met the criteria for deletion (**see 2.7.4**) are marked in red. Data derived from an earlier FISH study using YAC probes (marked in green) has been included for two patients (numbers 2 and 22 in this study, 21 and 15 in the previous study)[256]. In each case the figures refer to loss or retention not of the indicated probe but of a YAC hybridizing to the same region of 6q. In the earlier YAC study two distinct clonal deletions (21a and 21b) were found to coexist in one patient (number 2 in this study). The smaller of the two clones (21a) was present in approximately 95% of cells while 21b, which extended distally from 21a, was only seen in about 15% of cells. The additional PAC data presented in **Tables 3.3** and **3.8** confirms the existence of a minor clone in patient 2, although the relatively stringent scoring criteria (20% of analysed cells deleted) mean that not all probes within the second clone are marked as deleted. Thus RP1-293L8 has been scored as retained (marked in black) while RP1-179E13 was considered deleted (marked in red). The apparent existence of a non-contiguous deletion in patient 2 is therefore explained by the presence of a low level clone, with numbers of deleted cells close to the cut off level for this study. In all other cases, numbers of cells deleted were scored above the 20% level for all probes within the deleted region. In other words, no evidence for the existence of non-contiguous deletions was seen in this study.

All except 4 of the cases were deleted either for RP1-202B13 or the adjacent probe RP1-132E6; the region between these two probes was therefore a potential common region of overlap or most frequently deleted region among the cases analysed. The primary deletion data is presented in the form of a summary map with the potential common region of overlap highlighted (**Fig 3.2**). More detailed FISH analysis of the putative common region of overlap was carried out using probes from between RP1-202B13 and RP1-132E6 (**see 3.4.1**).

Probe	Patient or cell line (Number of cells deleted/number of cells retained)							
	1	2	3	4	5	6	7	8
RP1-71H19				00/10			00/15	00/37
RP1-260B21	10/02			00/10	05/00		10/04	
RP1-102H19				10/02				
RP1-67N15		14/06					10/02	
RP1-235B9	10/00		10/01	10/05		05/00		
P13743	00/10		08/04	10/04				
RP1-202B13	00/10	14/06	10/01	10/02	10/00	07/01		08/38
RP1-132E6		03/15	00/10	00/10	10/00		13/02	10/24
RP11-59I9	01/11		00/10	00/10	00/10	00/10		
RP1-429G5		7/23			00/10		13/02	09/28
RP1-111B22								
RP1-66H14			01/10	00/10			10/00	
RP1-240M13								
RP1-162C6								
RP1-59B14				00/10				
RP1-293L8		04/46						
RP1-179E13		07/43						
RP1-76H23							00/10	
RP1-171N11		00/20						00/30
RP1-172K10		01/19						
RP1-178D18								
RP1-237G20								
RP1-278N12								
RP1-137K2								
RP1-65A9								
RP1-167A14	00/12	00/20	00/20	00/20	00/20	00/20	00/20	00/50

Probe	Patient or cell line (Number of cells deleted/number of cells retained)							
	9	10	11	12	13	14	15	16
RP1-71H19		00/13	00/17	00/20		00/20	00/10	
RP1-260B21						10/06	10/00	10/00
RP1-102H19		00/10			10/00			
RP1-67N15	10/01	00/10		00/10			10/01	10/00
RP1-235B9	10/00	10/00	10/02	01/20		10/07	10/00	
P13743	10/03	10/01		10/09				
RP1-202B13	10/04	10/00				05/09	10/00	
RP1-132E6					10/00		10/00	
RP11-59I9		11/02	10/05	10/08	10/00	08/08		
RP1-429G5	11/03				10/00			
RP1-111B22					10/00			
RP1-66H14					00/10	07/15		10/00
RP1-240M13						05/13	10/00	
RP1-162C6		10/01				09/12	10/00	
RP1-59B14		10/00	10/07			00/10	00/10	10/00
RP1-293L8	11/11	00/10						
RP1-179E13	13/05	00/10						
RP1-76H23								
RP1-171N11	00/10	00/10		10/07			00/10	00/10
RP1-172K10		00/10						
RP1-178D18								
RP1-237G20								
RP1-278N12				10/17				
RP1-137K2								
RP1-65A9								
RP1-167A14	00/20	00/20	00/20	10/09	00/20	00/20	00/20	00/20

Probe	Patient or cell line (Number of cells deleted/number of cells retained)							
	17	18	19	20	21	22 *	23*	24
RP1-71H19	00/20	00/10		00/10	00/10	00/20	00/10	00/20
RP1-260B21	01/10	00/10						
RP1-102H19		00/10						
RP1-67N15	10/03			00/10	00/10			
RP1-235B9	10/02	10/00	00/10				00/14	
P13743								
RP1-202B13			10/00	00/10	00/10		00/05	
RP1-132E6			10/00	10/02	10/00	00/10		00/14
RP11-59I9	10/01	10/00	10/00			07/05	01/12	
RP1-429G5					10/01			
RP1-111B22				10/00			00/10	00/14
RP1-66H14		10/01					10/02	00/10
RP1-240M13								
RP1-162C6							10/04	
RP1-59B14	10/00	10/01	10/00				10/03	
RP1-293L8				10/00			00/09	
RP1-179E13				10/00			00/06	
RP1-76H23	10/01		10/00				00/10	00/10
RP1-171N11				01/11		08/06	00/12	
RP1-172K10	11/02			00/10		06/07		
RP1-178D18								00/10
RP1-237G20						07/08		
RP1-278N12	00/10		10/00			11/06		
RP1-137K2						11/07		10/10
RP1-65A9						06/05		
RP1-167A14	00/20	00/20	20/00	00/20	10/00	21/16	00/20	21/07

Table 3.3. Numbers of cells deleted for and retaining probes used in the primary FISH analysis of patients and cell lines with abnormalities of the q arm of chromosome 6.
* Deletion was associated with translocation in this patient. Probes scored as deleted are marked in red, data taken from an earlier study with YAC probes are marked in green.

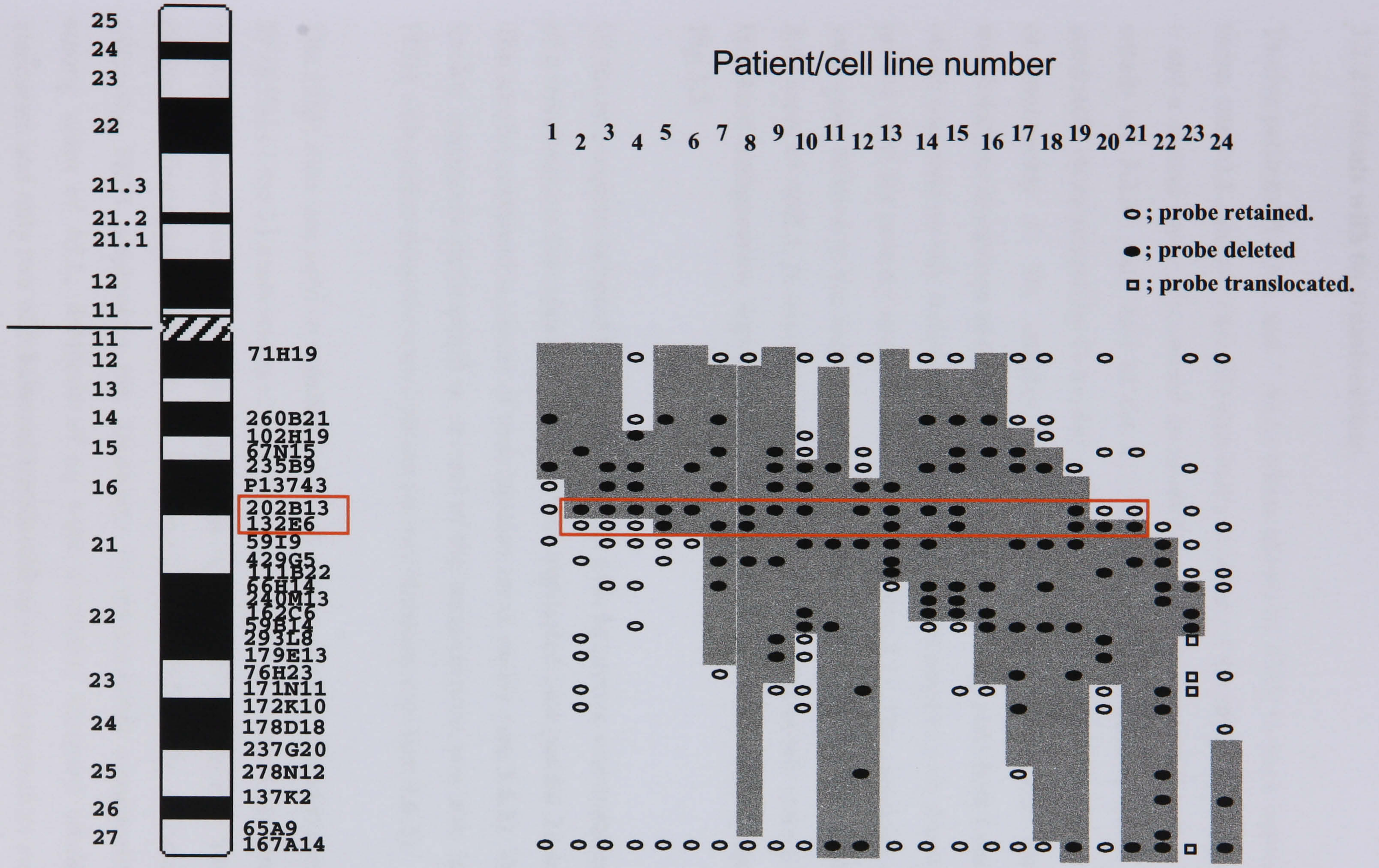


Fig 3.2 Summary of the primary FISH analysis of cases of acute leukaemia with deletions of 6q. The maximum extent of each deletion is indicated by gray infill and a putative common region of overlap between RP1-202B13 and RP1-132E6 enclosed within the red box.

3.2.2 Patients with 6q translocations.

Twelve patients (5 AML and 7 ALL) where shown by FISH to have translocations of 6q. Of these, one ALL patient (case 22) also had a deletion of the other homologue of chromosome 6 and a second case (23), carried an interstitial deletion associated with a translocation (for details see **3.2.3**). Only two of the ALL associated translocations (cases 26 and 29) were accurately characterized by G-banded analysis the remaining 5 were reported to be deletions or monosomy 6. By contrast 4 of the 5 cases of AML were classified as translocations/inversions and one was described as add(6q) (additional material of unknown origin was seen on 6q). Initially the translocations were mapped with the same panel of FISH probes used for primary analysis of deletions (**Table 3.2**). The positions of probes used in each case, relative to the break points, are presented in **Table 3.4** (cases of ALL) and **Table 3.5** (cases of AML). A summary map of translocation break points among both myeloid and lymphoid malignancies, highlighting regions selected for more detailed analysis, is shown in **Fig 3.3**.

Of the two regions selected for further analysis with 6q probes, one contained the break point of a translocation that appeared to have been duplicated (see patient 22 in **3.2.3** and **3.6.3**) The second contained a cluster of translocation break points (see **3.6.2**). The possibility that known oncogenes participated in several of the translocations was also investigated, using FISH with probes from the translocation partner chromosomes (see **3.6.1**).

The FISH data was used to redefine the break points of either translocations or deletions in 29 (85%) of the 34 cases analysed. A comparison of chromosome 6 abnormalities as defined by FISH analysis with the original karyotype is made for each case in **Table 3.6**. The high degree of discrepancy between 6q abnormalities as defined by G-banded analysis and following FISH emphasizes the limitations of conventional cytogenetics. In particular, among cases of ALL, deletions of 6q were invariably assigned breakpoints that were inaccurate and only two of 6 balanced translocations were recognized as such on G-banding. The results obtained in this study highlight the importance of using FISH, not only to define CDRs, but also prior to assigning clinical characteristics to recurrent cytogenetic changes.

Probe	Cases of ALL with translocations of 6q						
	22	23	25	26	27	28	29
RP1-71H19		C	C			C	C
RP1-260B21						C	
RP1-102H19						C	
RP1-67N15					C	C	
RP1-235B9		C					C
P13743*							
RP1-202B13		C			C		C
RP1-132E6					C	C	C
RP11-59I9	C	C			C	C	C
RP1-429G5			C				
RP1-111B22		C		C			
RP1-66H14		D	C	C			C
RP1-240M13							
RP1-162C6		D	C		C		
RP1-59B14		D	C	C		C	C
RP1-293L8		T	C	C			
RP1-179E13				C	C		
RP1-76H23		T	T	C	T	C	
RP1-171N11	C	T	T		T	T	
RP1-172K10	C		T	T		T	
RP1-178D18							
RP1-237G20	C				T		
RP1-278N12	C						
RP1-137K2	C						
RP1-65A9	C			T			
RP1-167A14	T	T	T	T	T	T	T
RP1-57M24	T			T			T

Table 3.4. Results of FISH analysis of ALL patients with translocations of 6q. Abbreviations are as follows: **C**: probe positioned on the centromeric side of the translocation break point. **T**: probe positioned on the telomeric side of the break point; **D**: probe deleted.

Probe	Cases of AML with balanced rearrangements of 6q					
	30	31	32a	32b	33	34
RP1-71H19	**C**		**C**	**C**	**C**	
RP1-260B21					**C**	
RP1-102H19						
RP1-67N15	**C**					
RP1-235B9	**T**					
P13743*						
RP1-202B13	**T**					
RP1-132E6						
RP11-59I9						
RP1-429G5		**C**	**C**	**C**		**C**
RP1-111B22						
RP1-66H14		**T**	**C**	**C**		
RP1-240M13						
RP1-162C6						
RP1-59B14					**C**	
RP1-293L8			**C**	**C**		
RP1-179E13			**T**	**C**		
RP1-76H23			**T**	**C**	**C**	
RP1-171N11		**T**	**T**	**T**	**C**	
RP1-172K10			**T**	**T**		
RP1-178D18						
RP1-237G20					**C**	
RP1-278N12						
RP1-137K2					**T**	
RP1-65A9						
RP1-167A14	**T**	**T**	**T**	**T**	**T**	**S**
RP1-57M24					**T**	

Table 3.5. Results of FISH analysis of cases of AML with translocations or inversions of 6q. Abbreviations are as in Table 3.4; in addition: **S**: probe split by translocation. 32a; translocation of 6q, 32b; pericentric inversion of 6q.

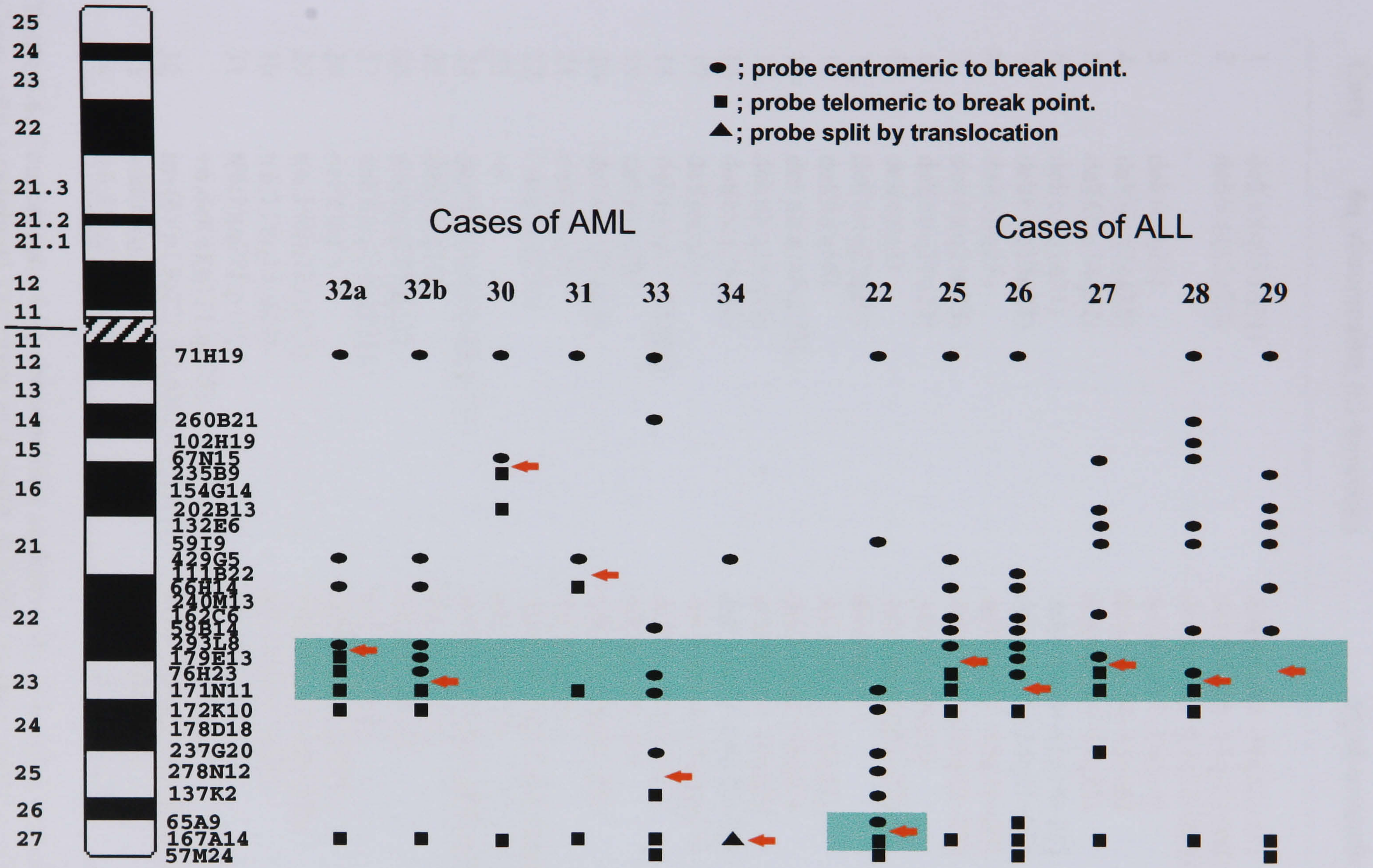


Fig 3.3 Summary of the primary FISH analysis of cases of acute leukaemia with translocations of 6q. The red arrows point to the position of translocation break points. Two regions selected for more detailed analysis are highlighted with blue infill.

Case	6q abnormality (G-banding)	6q abnormality (FISH)
1	del(6)(q?13q21)	del(6)(q11~14q15~16)
2	del(6)(q15q23)	del(6)(q11~14q16) (ml) del(6)(q11~14q22~23) (stl)
3	del(6)(q21)	del(6)(q11~14q16)
4	del(6)(q21q25)	del(6)(q14~15q16)
5	del(6)(q14q22)	del(6)(q11~14q21)
6	del(6)(q1q21)	del(6)(q11~15q16~21)
7	del(6)(q13q23)	del(6)(q11~14q21~23)
8	del(6)(q21)	del(6)(q11~15q21~23)
9	del(6)(q21q23)	del(6)(q11~15q22~23)
10	del(6)(q21q23)	del(6)(q15q22)
11	del(6)(q?)	del(6)(q15q22~27)
12	del(6)(q2?q2?)	del(6)(q15)
13	del(6)(q16)	del(6)(q11~15q21)
14	del(6)(q?15q2?3)	del(6)(q11~14q22)
15	del(6)(q13q21)	del(6)(q11~14q22)
16	del(6)(q13q22)	del(6)(q11~14q22~23)
17	del(6)(q21)	del(6)(q14~15q24~25)
18	del(6)(q13~15q21)	del(6)(q15q22~27)
19	del(6)(q22)	del(6)(q15~16)
20	del(6)(q21q23)	del(6)(q16q22~23)
21	del(6)(q21)	del(6)(q16)
22	?del(6)(q2?1)	del(6)(q16q27),t(6;?)(q27;?)+der?
23	-6	t(6;?)(q22;?)
24	der(6),t(1;6)(q?23;q?21)	der(6),t(1;6)(q?;q24~q25)
25	del(6)(q21)	t(6;?)(q22;?)
26	t(6;9)(q2?5;q22)	t(6;9)(q23;q?)
27	del(6)(q?13;q?21)	t(6;?)(q22;?)
28	del(6)(q?)	t(6;?)(q22~q23;?)
29	t(6;19)(q?21;p13)	t(6;19)(q22~q27;p?)
30	t(6;11)(q13;q23)	t(6;11)(q15;q23)
31	t(6;7)(q2?1;p15) +6,del(6)(q?21;q2?5),	t(6;7)(q21;p15) +6,del(6)(q?q?)
32	inv(6)(p13q22),t(2;6)(q3?1;q2?5)	inv(6)(p?q22),t(2;6)(q?;q22~q23)
33	add(6)(q23)	t(3;6)(q26;q25)
34	t(6;8)(q27;p11.2)	t(6;8)(q27;p11.2)

Table 3.6. Comparison of 6q abnormalities as defined by G-banded analysis and after FISH. Cases with a redefined karyotype as a result of FISH analysis with 6q locus specific probes are highlighted in red.

3.2.3 Complex rearrangements of chromosome 6.

Patterns of FISH signals consistent with complex rearrangements, that merit more detailed description, were seen in three of the cases included in the primary analysis.

Patient 22. This case was included in a previous published study from this laboratory in which deletions of 6q were mapped using FISH with YAC probes (patient 15[256]). YACs 860f10 and 36CB11 (equivalent to PAC RP1-32E6 and BAC RP11-59I9) defined the proximal boundary of deletion. In this study it was confirmed that the deletion encompassed the region containing clone 36CB11. FISH with probes between RP11-59I9 (6q21) and RP1-65A9 (6q27) resulted in a single green signal hybridising to a chromosome marked by the red 6q centromeric probe (RP1-71H19). As is typically seen with large deletions of 6q, these probes failed to hybridise to a second chromosome marked by the centromeric PAC. Unexpectedly, however, hybridisation of a probe mapped telomeric to RP1-65A9 (RP1-167A14), produced two green signals, neither of which were positioned on a chromosome marked by RP1-71H19. This pattern of FISH signals was open to more than one interpretation. In the least complex of these intrepretations, deletion of one homologue of 6q extend distally to include the telomere, and was therefore likely to be an unbalanced translocation. The second homologue of 6 underwent translocation with a break point between PACs RP1-65A9 and RP1-167A14. Following the translocation, the product containing the telomeric region of 6q was duplicated. The two chromosomes marked by RP1-167A14 differed in size suggesting that further rearrangement of one or both, in addition to translocation and duplication, had occurred. More complex models involving translocation of both homologues of 6q can also explain the pattern of signals seen.

Because the case evidently involved rearrangement of both copies of 6q, with the likely scenario being deletion of one homologue accompanied by translocation of the second, it seemed possible that the translocation break point would flag the site of a tumour suppressor gene. Paradoxically the apparent duplication of a translocation product was more characteristic of the type of gain of function changes associated with oncogenic fusion genes. It was decided to map the break point of the 6q translocation more precisely using PACs

localised to the region between RP1-65A9 and RP1-167A14. The results of analysis with further probes as well as FISH images illustrating the described signal pattern associated with this case are presented in **3.6.4**.

Patient 23. In this case PAC RP1-293L8 (6q22) and more telomeric probes hybridized to a chromosome of unknown origin and probes between the centromere and PAC RP1-111B22 (6q21) were retained on the derivative chromosome 6, consistent with the presence of translocation. However, intervening probes RP1-66H14, RP1-162C6 and RP1-59B14 were present on one homologue of chromosome 6 only, demonstrating that an interstitial deletion accompanied translocation in this case.

Patient 32. On G-banded analysis distinct clones with both an extra copy and structural rearrangements of chromosome 6 were recognised. In one clone, pericentric inversion of 6 was associated with deletion of a second homologue of 6q. A sub-clone with identical involvement of chromosome 6 but apparent duplication of the deletion was also reported. The deleted chromosome was not present but one homologue of 6 was involved in a translocation with chromosome 2 [t(2;6)(q3?1;q2?3)] in a second sub-clone. Evidence for the presence of an unbalanced t(6;6) was also found in the second sub-clone.

FISH with the 6q probes produced a pattern of signals consistent with the presence of both an inversion and a balanced translocation in approximately 50% of cells. The translocation and inversion were always co-existent and accompanied by a third, apparently normal, copy of chromosome 6. Evidence for interstitial deletion or unbalanced translocation of 6q was never seen in cells carrying the balanced rearrangements. However, a sub-clone with two normal copies and a large deletion of chromosome 6 was seen. Numbers of cells with del(6q) did not meet the scoring criteria adopted in this study so this case has not been included in the deletion analysis.

Examples of metaphase cells from patient 32 hybridised with PACs from the primary panel, are presented in **Fig 3.4**. The 6q inversion break points was found to fall between RP1-76H23 and RP1-171N11 and the t(2;6) translocation break point between PACS RP1-293L8

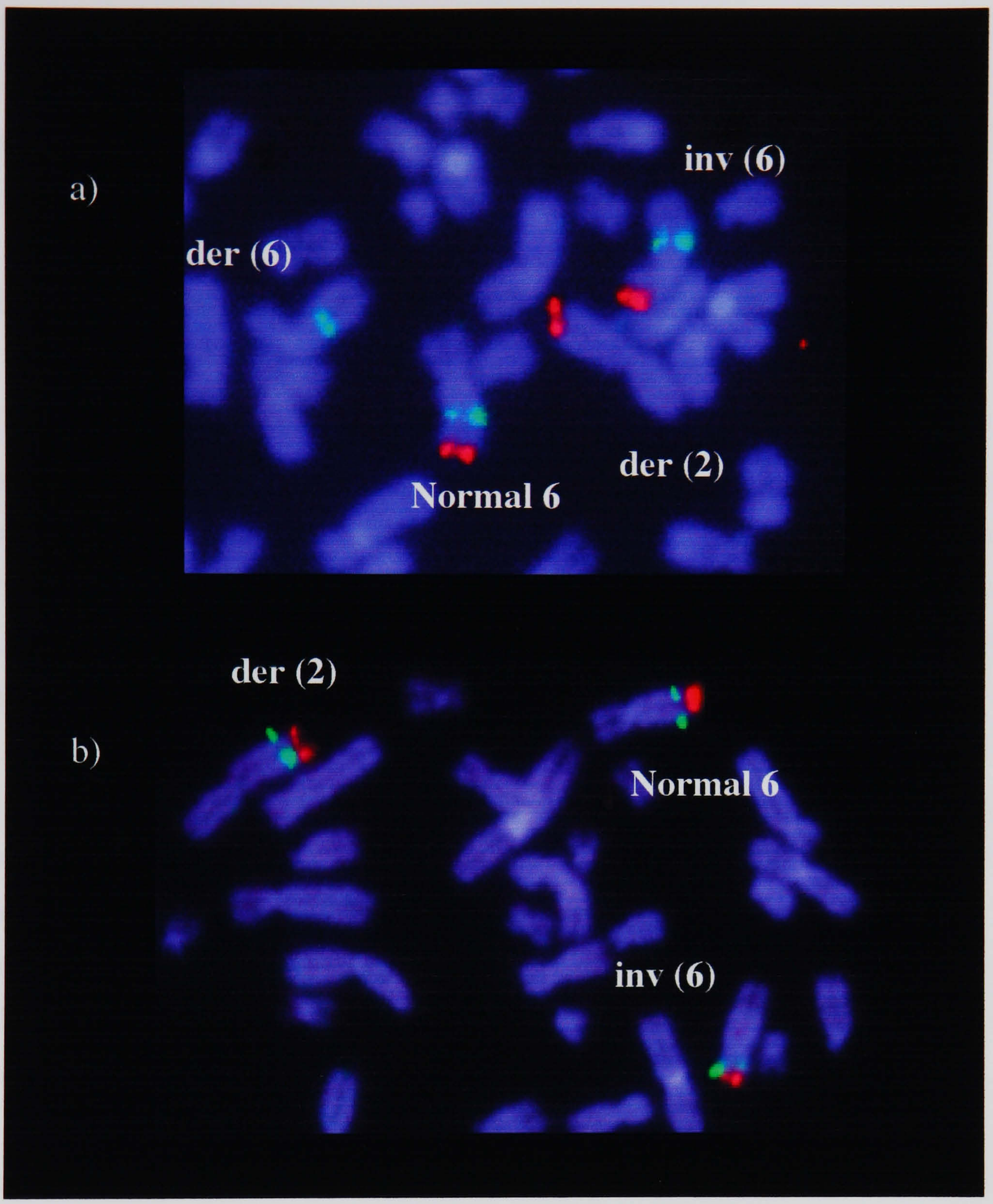


Fig 3.4 Example of FISH analysis of complex cytogenetic rearrangements including both translocation and inversion of chromosome 6 in patient 32. **a**) Red signal; RP1-167A14 (marking the the 6q telomeric region). Green signal; RP1-179E13 (hybridising to band q22 on chromosome 6). RP1-179E13 is positioned proximal and RP1-167A14 distal to the break points of both the t(2;6) and inv(6). **b**) Red signal; RP1-167A14 (marking the 6q telomeric region). Green signal RP1-171N11 (hybridising to band q23 on chromosome 6). Both RP1-167A14 and RP1-171N11 are positioned distal to translocation and inversion break points.

and RP1-179E13. The two balanced rearrangements therefore both interrupted the band 6q22-23 region within 7-11 Mb of each other. Translocation break points from 5 cases of ALL also fell within this region and additional PACs from between RP1-293L8 and RP1-171N11 were used to map the 7 rearrangements in greater detail (**see 3.6.2**).

3.3 Clinical details of Patients included in the study.

Clinical details (where available) for the patients and cell lines analysed by FISH are presented in table **3.7**. Of the 21 patients with deletions (including unbalanced translocations) of 6q, complete clinical data was obtained for all except three, of these two were cases of AML (21 and 24). Full clinical details were also unavailable for the three cell lines analysed (cases 5,13 and 16). The deleted patients represent a relatively homogeneous group in that all were children or young adults (age range 3-19 years) and of the 24 all except two were cases of ALL. Of these 11 were cases of T-Cell ALL, 6 had common ALL and two Pre-B ALL. The last three cases were described as ALL, unusual ALL and Null ALL/AML. White blood cell counts (WBCs) at diagnosis varied between 5.9 and $300x10^9$/L and there was an imbalance in the male/female ratio (17/7). Length of first remission varied between 1 and 111 months. Excluding two patients that remained in remission at the time the data was acquired the mean length of first remission was 35.4 months for the patients with 6q deletions.

Cases of ALL with balanced translocations of 6q (25-29 excluding patient 22 who carried both a deletion and a translocation) included two cases of common ALL and two of T-ALL with the remaining case being described as ALL. As for the deleted cases, there was a preponderance of male patients (4 male 1 female). WBCs were low for the cases of common ALL (3.5 and 2.3) but high for the T-Cell ALL patients (97.8 and 52.9). The mean remission time for four of the five patients was slightly higher than for the deleted cases (41.8 months) though the number of cases with translocations excluded the possibility of performing meaningful statistical analysis. Cases of AML with translocations of 6q had heterogeneous breakpoints (see Fig **3.3**) and no obvious common clinical features.

Case Number	Age at Diagnosis	sex	WBC	Disease sub type	Length of 1st remission
1	12	F	272.0	Common ALL (L1)	22
2	13	F	8.8	Pre-B ALL	10
3	13	M	51.0	T-Cell ALL (L1)	13
4	10	M	166.4	T-Cell ALL	24
5*	16	F		T-Cell ALL	
6	8	M	282.0	T-Cell ALL (L1)	12
7	15	M	200.0	T-Cell ALL	1
8	15	M	300.0	T-Cell ALL	36+
9	13	M	44.5	T-Cell ALL (L1)	31
10	4	M	13.9	Common ALL (L1)	34
11	3	M	5.9	Unusual ALL (L1)	95
12	4	M	16.1	Common ALL	27+
13*	19	M		T-Cell ALL	
14	16	M	104	T-Cell ALL	9
15	8	M	15.7	T-Cell ALL (L1)	26
16*	5	F		T-Cell ALL	
17	4	F	9.9	Pre-B ALL (L1)	111
18	11	M	8.5	Common ALL (L1)	102
19	10	M	35.0	Common ALL (L1)	25
20		F		ALL	
21		M		AML	
22	10	M	7.3	Null ALL/AML	39.5
23	8	F	17.6	Common ALL (L1)	12
24		M		AML	
25		M		ALL	
26	16	M	3.5	Common ALL	21.5
27	10	M	97.8	T-Cell ALL	33
28	2	M	52.9	T-Cell ALL	77
29	10	F	2.3	Common ALL	36
30	13	F	9.0	AML (M4)	1.5
31	1	F	15.4	AML (M2)	2
32		M		AML	
33		F		AML (M5)	
34	15	M	12.0	AML (M4)	11

Table **3.7** Clinical data for patients with abnormalities of 6q analysed by FISH. Age (years), WBC ($x10^9$/L), length of first remission (months). * indicates cell line.

3.4 Further analysis of deleted Cases.

FISH analysis with the primary probes demonstrated that in all, except for four cases, deletions extended into the region between PACs RP1-202B13 and RP1-132E6. Three cases (2,3 and 4) were deleted for RP1-202B13 but not RP1-132E6 and two cases (20 and 21) retained RP1-202B13 and showed loss of RP1-132E6. It was therefore of interest to map, in greater detail, the extent of deletion in these patients. Firstly to establish if there was a common overlapping region of deletion between the five cases and secondly to investigate the possibility that any of these deletions shared a common break point.

3.4.1 Secondary FISH analysis of deleted cases.

A further 6 PAC probes localised between RP1-202B13 and RP1-132E6 were used in FISH experiments to more accurately map the position of deletion break points in the five cases. The probes used, their corresponding genetic markers and HGMP map positions are presented in **Table 3.8**. Numbers of cells retaining or deleted for the probes used in each patient are detailed in **Table 3.9**.

On further analysis each of the five deletions overlapped to give a common deletion region (CDR) of 4.5 Mb, between PACs RP1-48F9 and RP1-299C21 (**Fig 3.5**). At least 4 different break points were shared between the five cases suggesting that there was no common sequence underlying the mechanism leading to deletion in these cases. Potentially a common break point forms the distal boundary of deletion in patients 3 and 4. However, the region between RP1-299C21 and RP1-132E6 is large (not less than 1Mb) and at the time of analysis was partly uncloned. Because of limitations in the availability of both suitable clones and patient material, further analysis to determine whether the two cases shared a common break point was not performed.

Probe	Chromosome 6 map position	Cytogenetic band	Marker
RP1-202B13	100600-100700	6q16	D6S501
RP1-48F9	101700-101900	6q16	D6S1510
RP1-121G13	103550-103650	6q16	D6S1409
RP1-204F17	104700-104800	6q16	D6S1543
RP1-256E11	105250-105350	6q16	D6S1642
RP1-201G21	105900-106050	6q16	D6S283
RP1-299C21	106350-106450	6q16	D6S1692
RP1-132E6	108150-108300	6q16	D6S447

Table 3.8. Probes used for FISH analysis of the common deleted region, with corresponding HGMP chromosome 6 map positions, band assignments and genetic markers.

Probe	Patient or cell line (Number of cells deleted/number of cells retained)				
	2	3	4	20	21
RP1-202B13	14/06	10/01	10/02	00/10	00/10
RP1-48F9				10/00	00/10
RP1-121G13				09/01	10/00
RPI-204F17	10/02				10/00
RP1-256E11	10/02				
RP1-201G21	10/03	10/03			
RP1-299C21	03/10	11/01	10/03		
RP1-132E6	03/15	00/10	00/10	09/02	10/01

Table 3.9. Secondary FISH analysis of patients 2, 3 and 4 (deletions ending distally between RP1-202B13 and RP1-132E6) and patients 20 and 21 (proximal ends of deletions falling between RP1-202B13 and RP1-132E6). Numbers of metaphase cells showing deletion/retention of probes positioned between 202B13 and 132E6. Probes scored as deleted are marked in red, retained probes in black. Data marked in green refers to analysis with YAC 830d9 performed in an earlier FISH study[256]

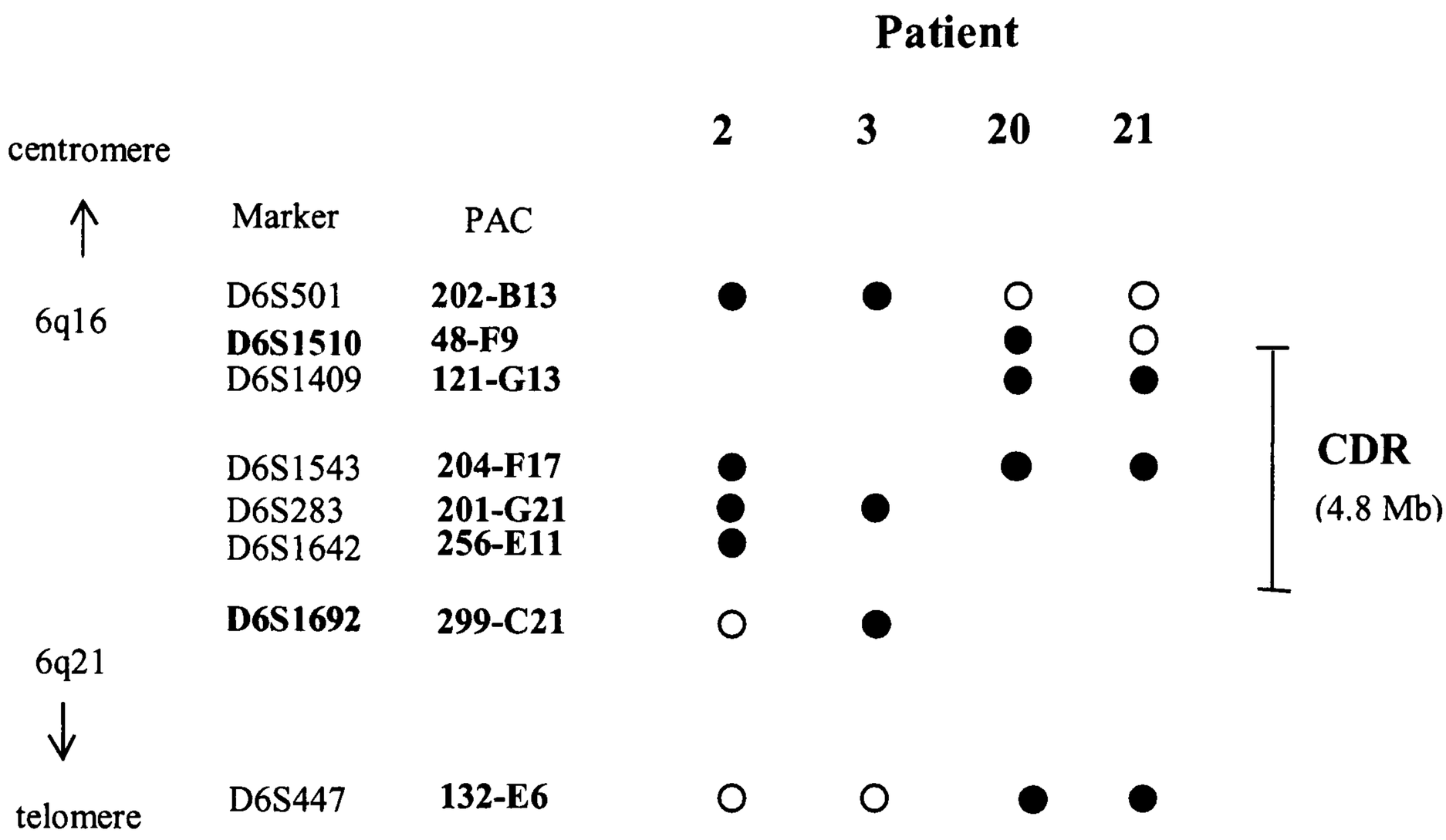


Fig 3.5. Detailed FISH map of the region between PACs 202-B13 and 132-E6 in patients with leukemia related deletions of 6q. Open circle (probe retained), Filled circle (probe deleted). A common region of deletion was defined between PACs 48-F9 (marker D6S1510) and 229-C21 (marker D6S1692).

3.4.2 Gene content of the new CDR.

Analysis of the region between RP1-299C21 and RP1-48F9 using the ENSEMBL and ACEDB human genome browsers revealed the presence of 16 genes. At the time the search was conducted (March 2002) fully sequenced clones accounted for 98-99% of the region. Additional exons either associated with one of the recognized genes or with as yet unrecognized genes in the region were also predicted by the presence of open reading frames (Gene scan predicted) and or of NCBI curated cDNA sequences. Of the 16 genes, 6 had been previously characterized, 8 corresponded to full length or near full length cDNA or protein sequences and 2 were predicted on the basis of similarity to ESTs or known protein sequences. The positional relationship between the recognized genes, predicted genes and sequence segments is summarized in **fig 3.6**. Further information about the size, number of exons and function or predicted function of the genes is presented in **tables 3A.1** and **3A.2** (Appendix 3).

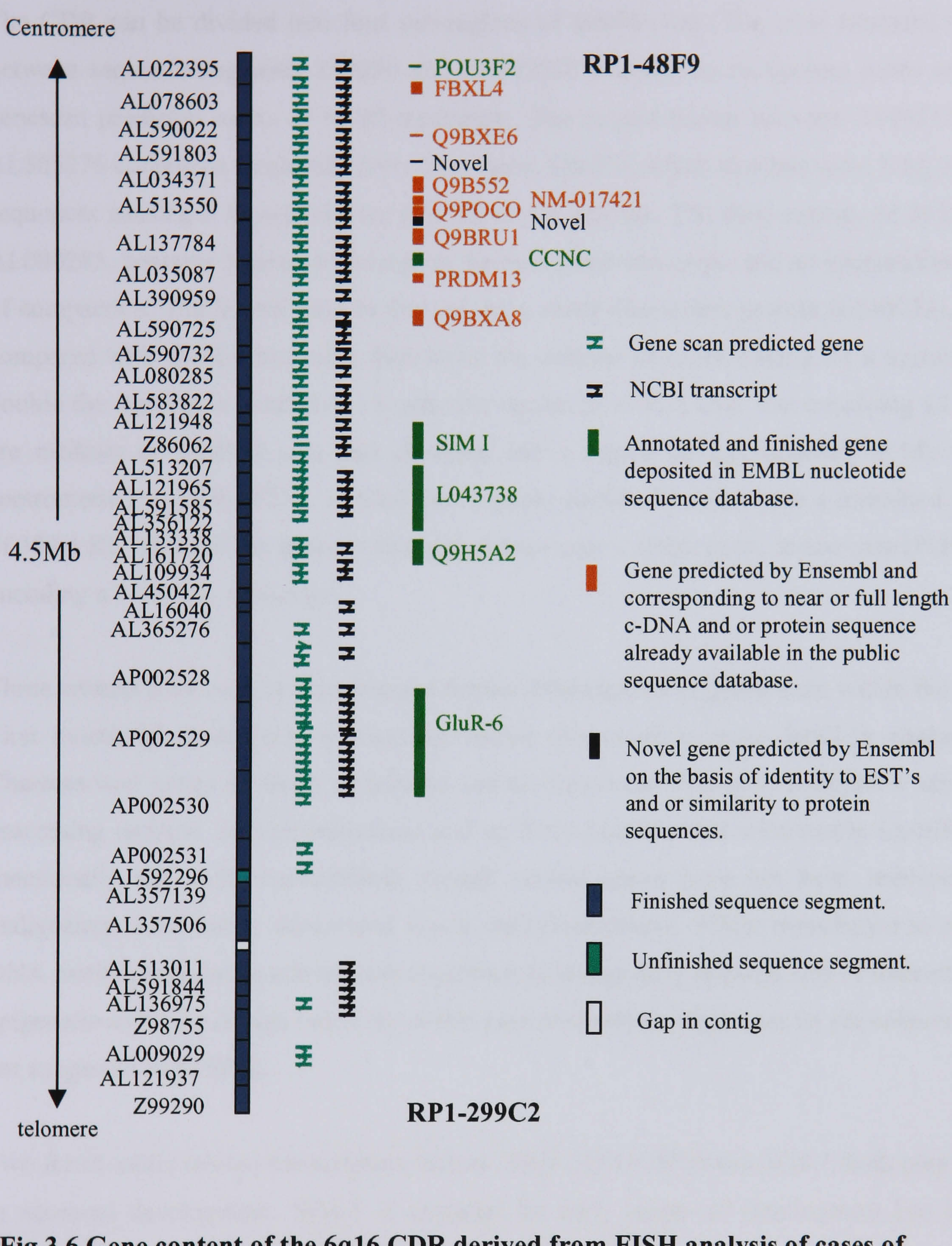


Fig 3.6 Gene content of the 6q16 CDR derived from FISH analysis of cases of acute leukaemia with cytogenetic deletion of chromosome 6. Showing the Positional relationship between sequenced segments, genes, predicted genes and transcripts

The CDR can be divided into four sub-regions of similar size. The most telomeric region between sequence segments Z99290 and AP002530 contains no recognised genes and few genescan predicted exons or NCBI transcripts. The second region between AP002530 and AL365276 contains a single relatively large gene, *GluR-6*, which also has some long intronic sequences causing it to extend over 670 Kb of the genome. The third region, AL365276 to AL080285, contains 3 genes with large to medium sized transcripts and an intermediate level of compaction. The largest gene in this region, a small ribonuclear protein (L043738), when compared with *GluR-6* has more than twice the number of exons coding for a transcript of double the size but is localized to a genomic region of similar size. The remaining 12 genes are medium or small in size and clustered into a region of approximately 1 Mb in the centromeric part of the CDR. In total the 12 genes encode 70 exons with a combined length of 304.4 Kb. Three of the genes in this region have only a single exon, in one case (POU3F2) encoding a 1329 b.p. transcript.

Three criteria were used to prioritise the further investigation of genes from within the CDR. First functional characteristics were considered (discussed in more detail in **chapter 4**). Characterised genes or those containing known functional domains, included a ubiquitin processing enzyme, an oxidoreductase and an RNA helicase that were newly identified so functionally not well characterized, though related genes have not been implicated in malignancy. Also newly discovered was a methyltransferase. While disturbance to normal DNA methyletransferase activity can contribute to malignancy through loss of imprinting or epigenetic silencing of tsgs (see **1.5**), in this case methylation targets are as yet unknown and not suggested to be DNA .

Two functionally related transcription factors, *BRN-2* (*POU3F2*) and *SIM-1*, both play a role in neuronal development. SIM-1 is essential for early stages of development but is also required to maintain expression of *BRN-2* that in turn directs terminal differentiation[263]. BRN-2 is also required for melanocyte development and SIM-1 for differentiation of neuronal cells that regulate aspects of behavior such as appetite. A patient with a constitutional translocation involving *SIM1* developed obesity but not malignant disease[264].

A second gene involved in appetite regulation, *MCH2* is a G-protein coupled receptor activated by melanin concentrating hormone[265].

The last two characterized genes, *CCNC* and *GluR-6*, are both functionally interesting candidate tsgs. *GluR-6* because it encodes a transmembrane receptor sub-unit that is involved in the transduction of pro-apoptotic signals and CCNC because it forms part of a mediator complex that modulates RNA polymerase II activity and has been implicated in the negative regulation of cell growth (for further discussion and references see **5.1**).

In addition to considering the functional properties of encoded proteins, knowledge of the tissue specific expression of genes from within the CDR could potentially be used to prioritise candidate TSGs. Expression patterns of many of the genes within the CDR were unclear but *SIM I* and *GluR-6* have been associated exclusively with a role in central nervous system. *BRN-2 (POU3F2)* expression has been reported only in brain and melanocytes. *MCH2* (Q9BXA8) was expressed at highest levels in various regions of the brain. However, among 19 other tissues investigated, lower numbers of transcripts were detected in lymphocytes, the intestine, adipose and prostate tissue[265]. *CCNC* was transcribed at highest levels in pancreas, heart, liver, skeletal muscle and kidney but a functional role for the protein in haematopoietic cells has also been reported[266].

As a third means of focusing the search for a TSG on 6q the data was compared with previously published CDRs derived either from FISH or LOH analysis of leukemia and lymphoma patients (see **3.4.3**).

3.4.3 Comparison with previously published CDRs.

Genetic markers, defining the proximal and distal boundaries of 6 previously published 6q CDRs, were positioned on Sanger Centres's PAC and BAC contigs by searching the ACEDB and NCBI databases. The positions of the 6 published CDRs and the new FISH CDR were then mapped relative to each other (**Fig 3.7**). The CDR obtained in this study overlapped with four of the six previously published. In one study distinct proximal and distal CDRs,

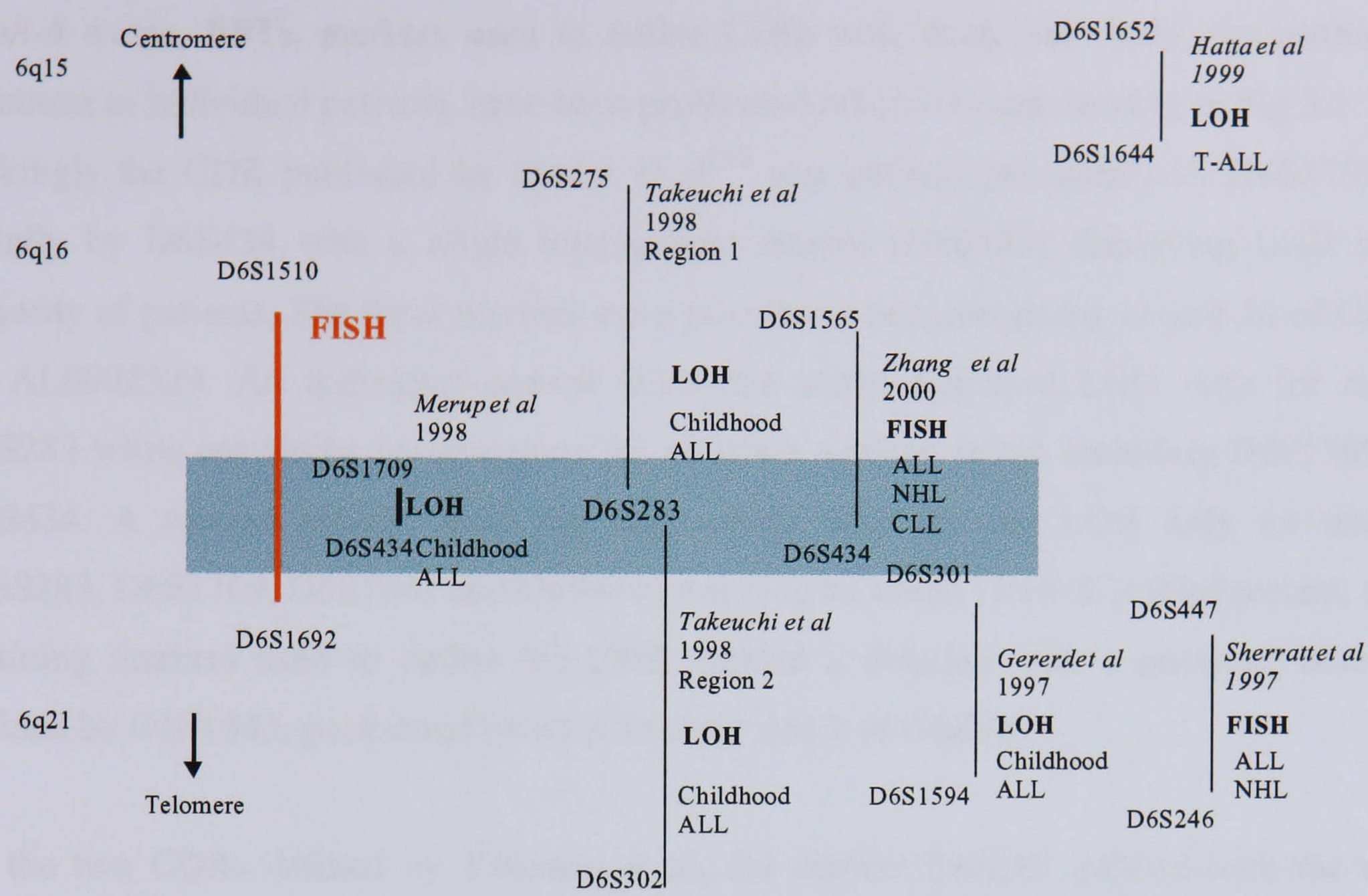


Fig 3.7 Relative position of CDRs derived from FISH or LOH analysis of lymphoid malignancies in relation to the new FISH CDR. 5 of the 8 CDRs overlapped to give a consensus region centred on marker D6S283 (highlighted by the blue box).

both of which overlapped with our own region, were published (Takeuchi et al)[259]. The two CDRs also overlapped with two additional published CDRs to give a consensus region centred on marker D6S283 and coinciding with the second of the two sub-regions described in **3.4.2**. The sub-region was composed of 4 fully sequenced segments (AP002531, AP002530, AP002529 and AP002528) and contained a single recognized gene, *GluR-6*. Using the BLAST search facility of NCBI the markers used to define the published CDRs and individual exons from the publicly available *GluR-6* cDNA sequence (NCBI accession # NM_021956) were mapped on the four sequence segments. NCBI EST databases were also compared for matches with the four sequences.

GluR-6 exons, ESTs, markers used to define CDRs and, from one study, the position of deletions in individual patients, have been positioned relative to one another in **Fig 3.8**. Most strikingly the CDR published by Merup et al[258] was defined proximally by D6S1709 and distally by D6S434 with a single intermediate marker (D6S283) displaying LOH in the majority of patients. The three markers were positioned between exons 11 and 14 of *GluR-6* on AL0002529. An individual patient from this study displayed LOH only for marker D6S283 while remaining heterozygous for all other markers tested including D6S1709 and D6S434. A second patient, from the same study, also showed LOH only for markers (D6S283, D6S1709, D6S1642 and D6S449) mapping to within *GluR-6. A* third patient, while retaining markers used to define the CDR, carried a deletion with a proximal boundary defined by D6S1543, positioned between exons 1 and 2 of *GluR-6.*

Of the two CDRs defined by Takeuchi et al, the marker D6S283 defined both the distal boundary of the more centromeric and the proximal boundary of the more telomeric. Both CDRs therefore encompassed exons of *GluR-6.* Consequently, loss of function of GluR-6 could result from all of the deletions observed in this study, obviating the need to postulate the existence of two distinct TSGs on 6q. The CDR from a third study also included exons 1-13 of *GluR-6* (Zhang et al)[257].

A detailed blast search of the four sequenced segments revealed 18 ESTs. On the basis of sequence homology to common repeat sequences, lack of evidence for splicing and lack of cross species conservation, it seemed unlikely that any of the ESTs represented functional genes.

In summary, there was no single clear region of overlap between the new CDRs and those published by other groups. However detailed comparison between published leukemia associated deletions of 6q and genes located within the new CDR identified *GluR-6* as the most promising candidate tumour suppressor.

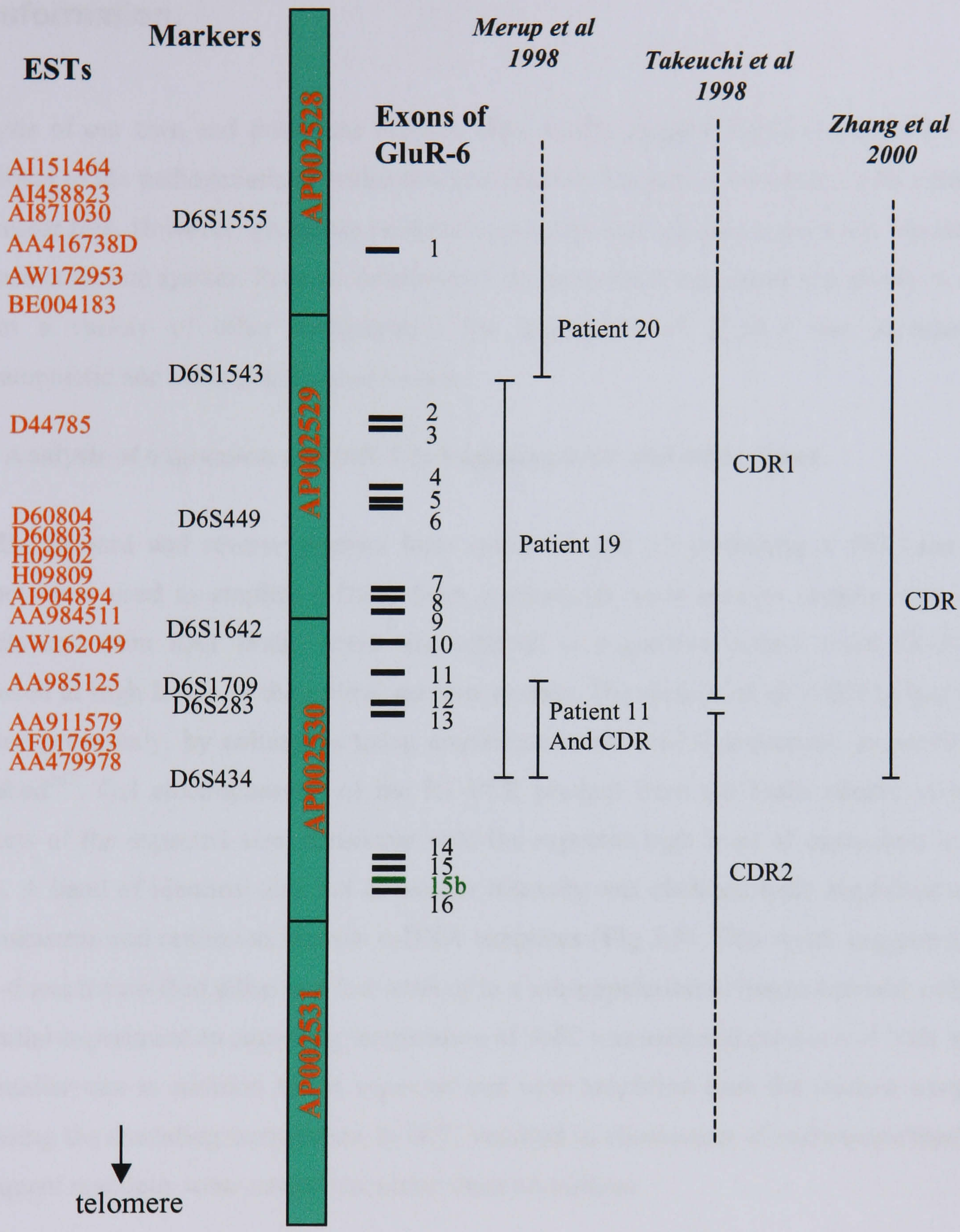


Fig 3.8 The positional relationship between sequenced fragments, ESTs, exons of GluR-6, and published CDRs (and deletions in individual patients) derived from LOH analysis of cases of ALL.

3.5 Investigation of the potential role of *GluR-6* in malignant transformation.

Analysis of our own and published deletion data clearly suggest that loss of *GluR-6* may contribute to the pathogenesis of leukaemia and GluR-6 function is consistent with a tumour suppressor role. However, glutamate receptor expression had not previously been reported in the haematopoietc system. Because deletions of chromosome 6 are common not only in ALL but in a variety of other malignancies the expression of *GluR-6* was assessed in haematopoietic and several additional tissues.

3.5.1 Analysis of expression of *GluR-6* in haematopoietic and other tissue.

Initially forward and reverse primers from exons 13 and 15 producing a 469 base pair product were used to amplify c-DNA from a remission bone marrow sample, two ALL patients and from RAT brain. Brain was selected as a positive control since *GluR-6* is expressed at high levels in the central nervous system. The quality of all c-DNAs had been checked previously, by colleagues using amplification for *G6PD* sequences, as previously described[267]. Gel electrophoresis of the RT-PCR product from the brain sample revealed products of the expected size, consistent with the expected high level of expression in this tissue. A band of identical size but of weaker intensity was obtained from amplification of the leukaemia and remission marrow c-DNA templates (**Fig 3.9**). This result suggested that *GluR-6* was transcribed either at a low level or in a sub-population of haematopoietic cells. In this initial experiment an annealing temperature of 66^0C was used and products of both larger and smaller size in addition to the expected one were amplified from the marrow samples. Increasing the annealing temperature to 68^0C resulted in elimination of extraneous bands so subsequent reactions were carried out under these conditions.

c-DNA from 5 further remission bone marrow samples, a T-ALL cell line (CEM), a B cell ALL cell line (REH) and a presentation sample from a CML patient were then amplified with the same *GluR-6* primers. Products of the expected size were obtained from all the remission samples, though with some variation in band intensity (possibly relating to differences in the

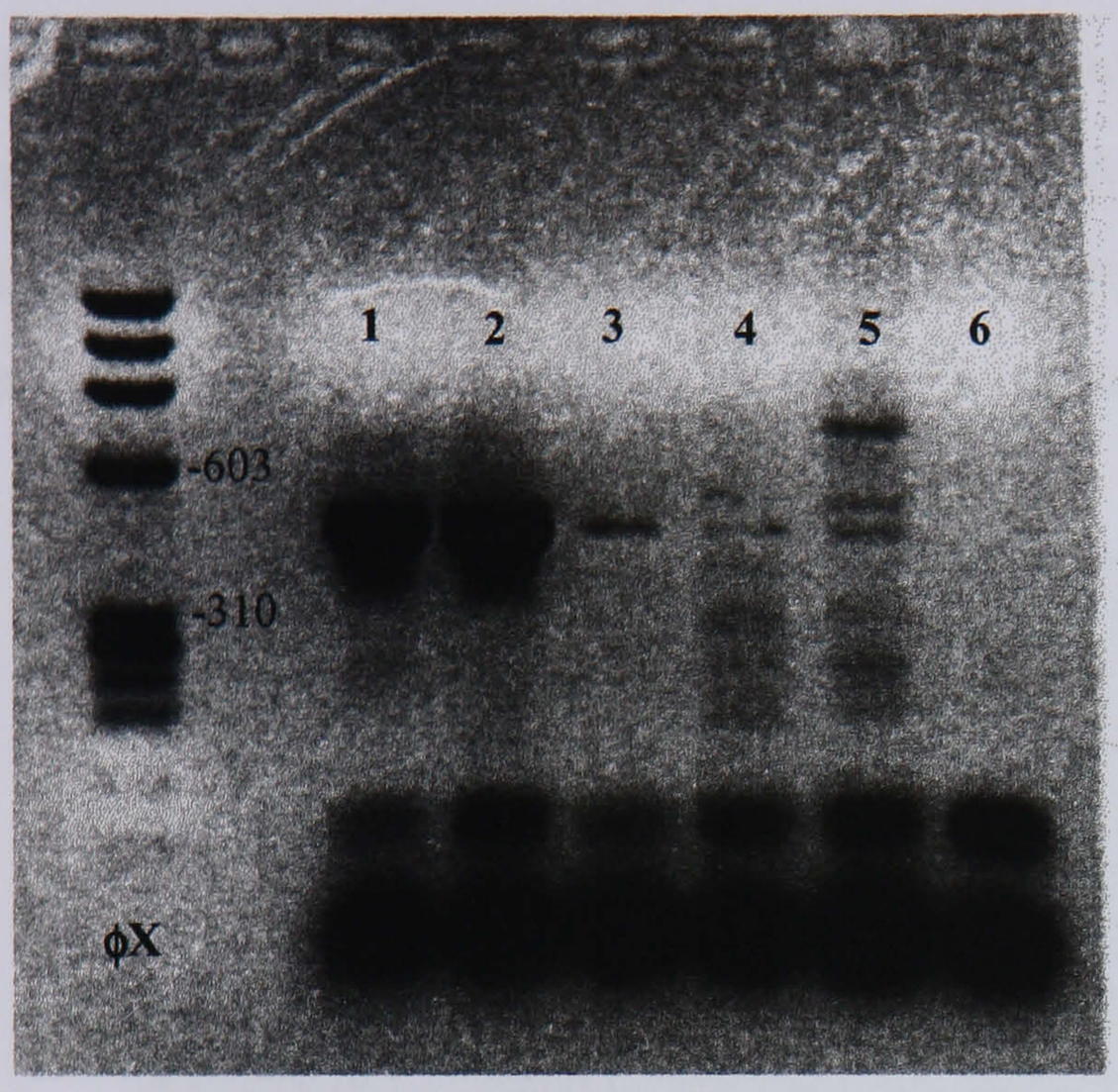


Fig 3.9 PCR with paired GluR-6 specific primers and cDNA from brain, normal and leukemic bone marrow. (1 and 2) rat brain, (3) remission bone marrow sample, (4) B-ALL patient (5) T-ALL patient (6) H_2O.

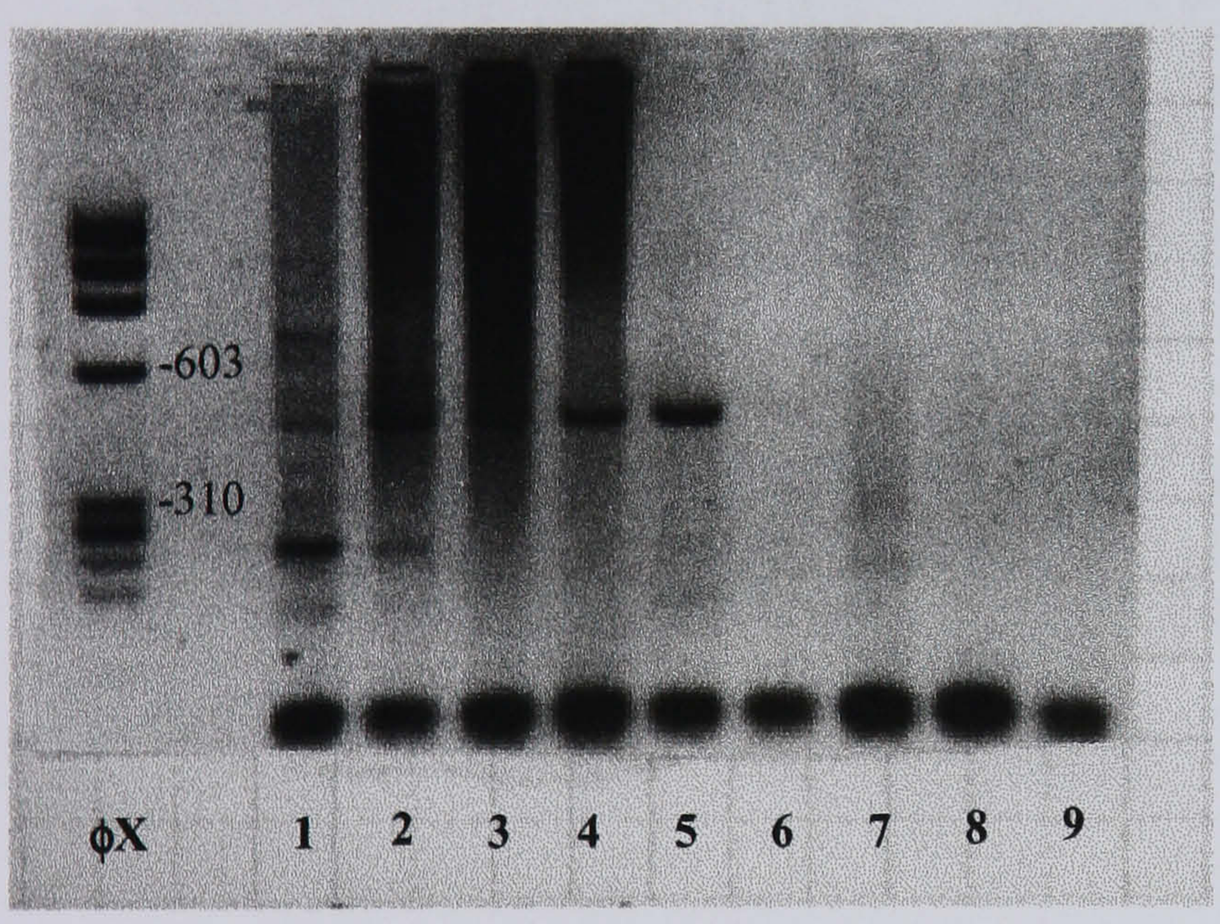


Fig 3.10 PCR with paired GluR-6 specific primers and cDNA from cell lines a CML patient and normal marrow. (1-5) remission bone marrow sample, (6) CML presentation sample, (7) REH; B-cell ALL cell line, (8) CEM; T-ALL cell line, (9) H_2O

quality of analysed cDNA). No visible product resulted from amplification of the CML patient or cell line c-DNA (**Fig 3.10**). A further 2 remission bone marrow samples, 2 lymphoid cell lines (CEM and NC37), a CML cell line (K562), 2 ALL patients, 2 CLL patients and 5 AML patients were then tested with the *GluR-6* primers. Clear bands of the expected size were seen in both remission samples, the lymphoid cell lines, the cases of ALL and one of the AML patients (**Fig 3.11**). Amplification from the other samples produced either very weak or no visible bands. Though not fully consistent, these results suggested that

expression of *GluR-6* is associated with lymphoid rather than myeloid lineages. It should be noted that CEM gave a positive result although it had been negative in the previous experiment using the same primers. The inconsistent results obtained with these primers suggested either that there were technical problems associated with their use or that *GluR-6* mRNA were present at a level close to the threshold of sensitivity of the technique applied.

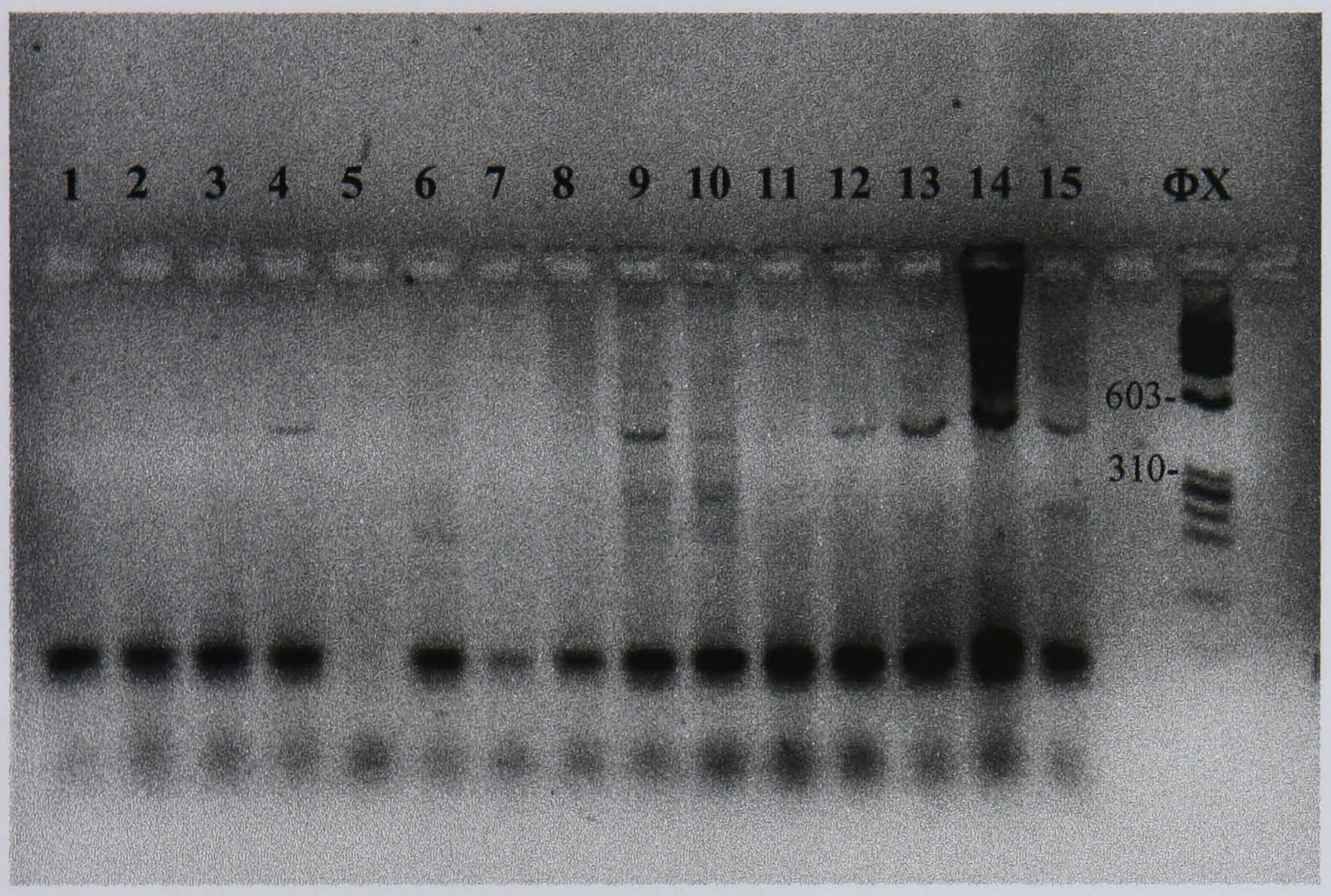


Fig 3.11 RT-PCR analysis of marrow samples from leukaemia patients and cell lines using primers specific for *GluR-6*. (1) H_2O, (2-6) cases of AML, (7-8) cases of CLL, (9-10) cases of ALL, (11) K562, (12) CEM, (13) NC37, (14-15) remission samples.

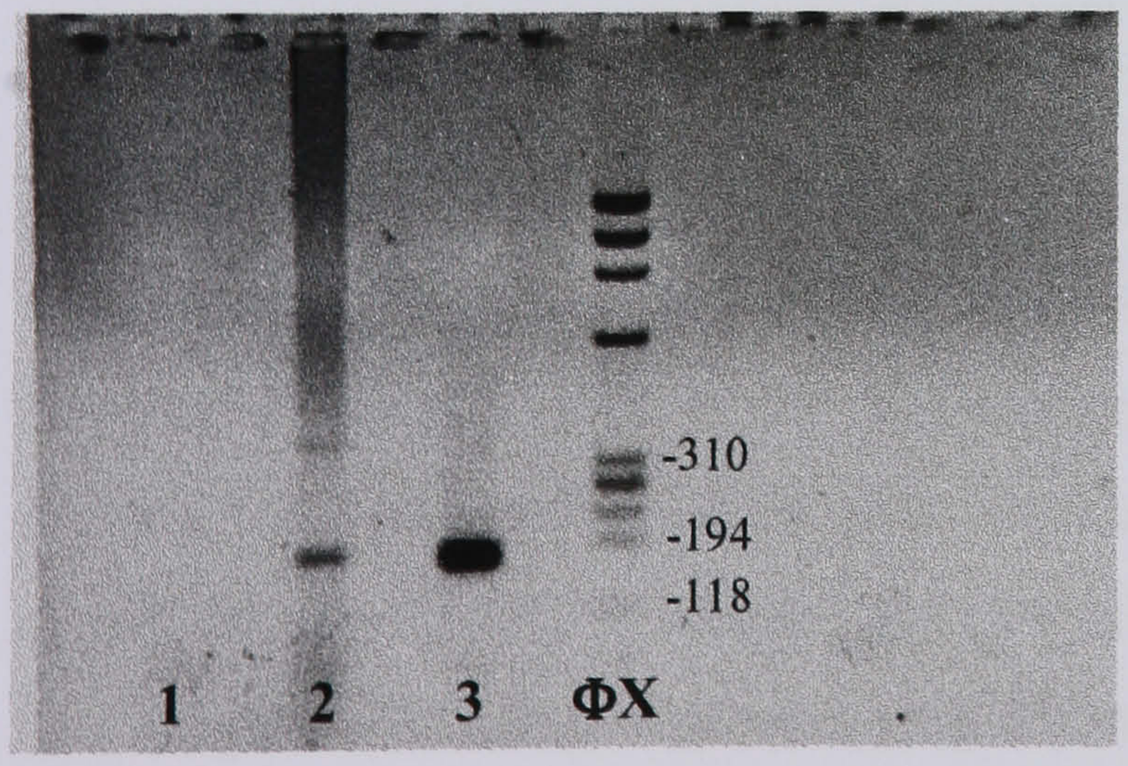


Fig 3.12 RT-PCR analysis with the new GluR-6 (2) primers. (1) H_2O (2) remission bone marrow, (3) rat Brain.

In an effort to improve the efficiency of detection of GluR-6 transcripts, new primers were designed from exons 1 and 2, the expected product size was 173 base pairs. Amplification of brain and remission bone marrow cDNA templates resulted in products of the expected size (**Fig 3.12**), and consistent with the earlier findings GluR-6 transcripts appeared significantly more abundant in the brain sample.

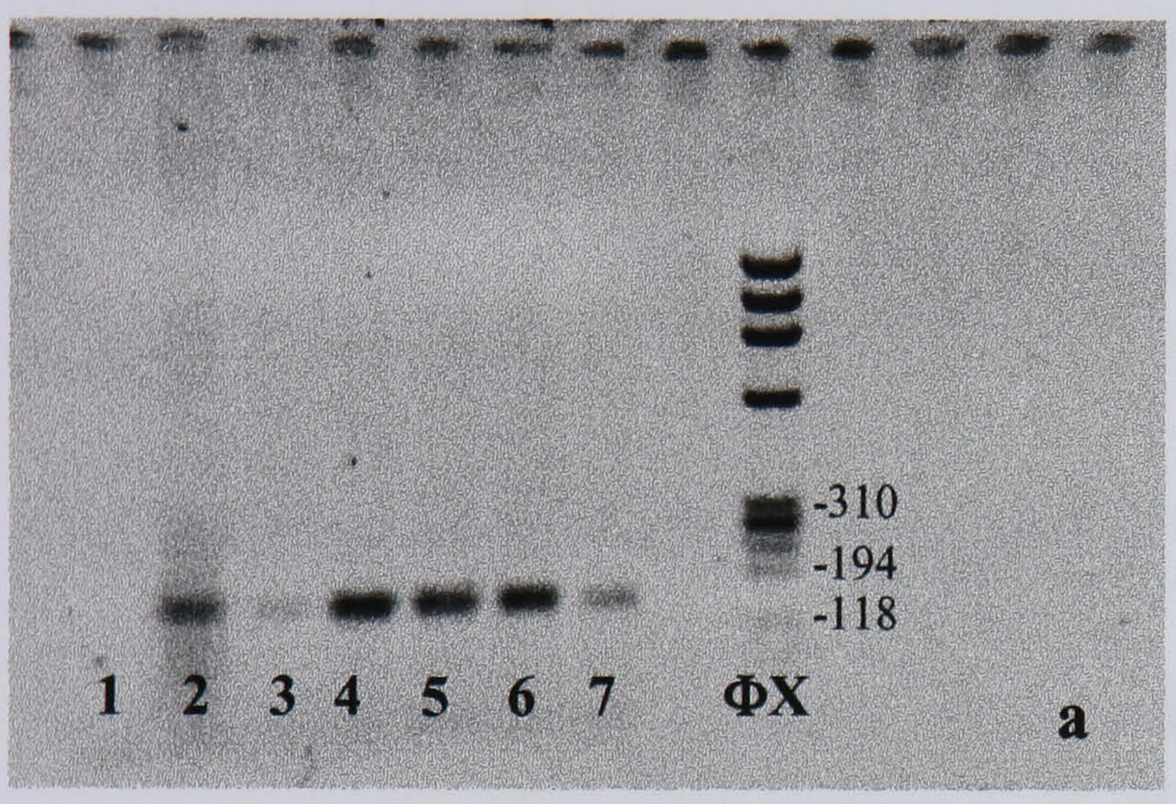


Fig 3.13 RT-PCR analysis of Different tissues with (a) *G6PD* and (b) *GluR-6* (2) primers.
(1) H_2O, (2) B-cells, (3) liver, (4) lung, (5) trachea, (6) kidney, (7) heart.

cDNA was then prepared from EBV transformed B-lymphocyte RNA, and commercially available liver, lung, trachea, kidney and heart RNA. The quality of cDNAs samples was assessed by amplification of *G6PD* sequences prior to amplification using the new *GluR-6* primers. cDNA prepared from liver and heart appeared of low quality, as judged by amplification with *G6PD* primers, and no visible product resulted from amplification of these two samples with *GluR-6* primers (**Fig 3.13a**). PCR with *G6PD* primers and the remaining four cDNAs resulted in similar levels of amplification, consistent with high quality cDNA

templates in each case. By contrast, considerable variation was seen in the quantity of product resulting from amplification of these templates with *GluR-6* primers (**Fig 3.13b**). Based on this experiment, GluR-6 transcripts were several-fold more abundant in kidney than in lung, with B-cells and trachea showing evidence for intermediate levels of expression.

In summary; a visible RT-PCR product was consistently obtained from normal bone marrow samples using *GluR-6* primers and one-round PCR amplification. Analysis of patient and cell line cDNA suggested that expression might be confined to the lymphoid lineages. Evidence for variable levels of *GluR-6* expression was also found in several non-haematopoietic tissues.

3.5.2 Mutation analysis of *GluR-6* in patients with large deletions of the region.

The possibility that large deletions of 6q contribute to the pathogenesis of leukaemia by unmasking inactivating point mutations of *GluR-6* was next investigated. Genomic DNA from ALL patients or cell lines with evidence for deletions that included all or part of *GluR-6* was obtained from two sources. Seven cases were analysed by FISH, either with PACs in the present study or with YACs in an earlier study from this department (cases 7-14). Seven further cases had been found to display LOH of markers in the region (cases 1-7) (analysis conducted by Dr A.Sorour in this department, personal communication).

PCR reactions using primers designed to amplify each of the 16 known exons of *GluR-6* were first performed with a remission bone marrow DNA sample. Products of the expected size, without significant secondary bands, were obtained for all exons except 6 12 and 15 (**Fig 3.14a**). On repeating the PCR reaction a satisfactory product was obtained using primers for exon 12 and 15 but not for exon 6. However, this was shown to be due to poor amplification ability of these primers. A new set of primers flanking exon 6 amplified a suitable product (**Fig 3.14b**). Evidence for the existence of a 17th exon (15b) was published in the course of the analysis of patient DNA samples[268]. Primers flanking the additional exon were designed and tested retrospectively (**Fig 3.14c**).

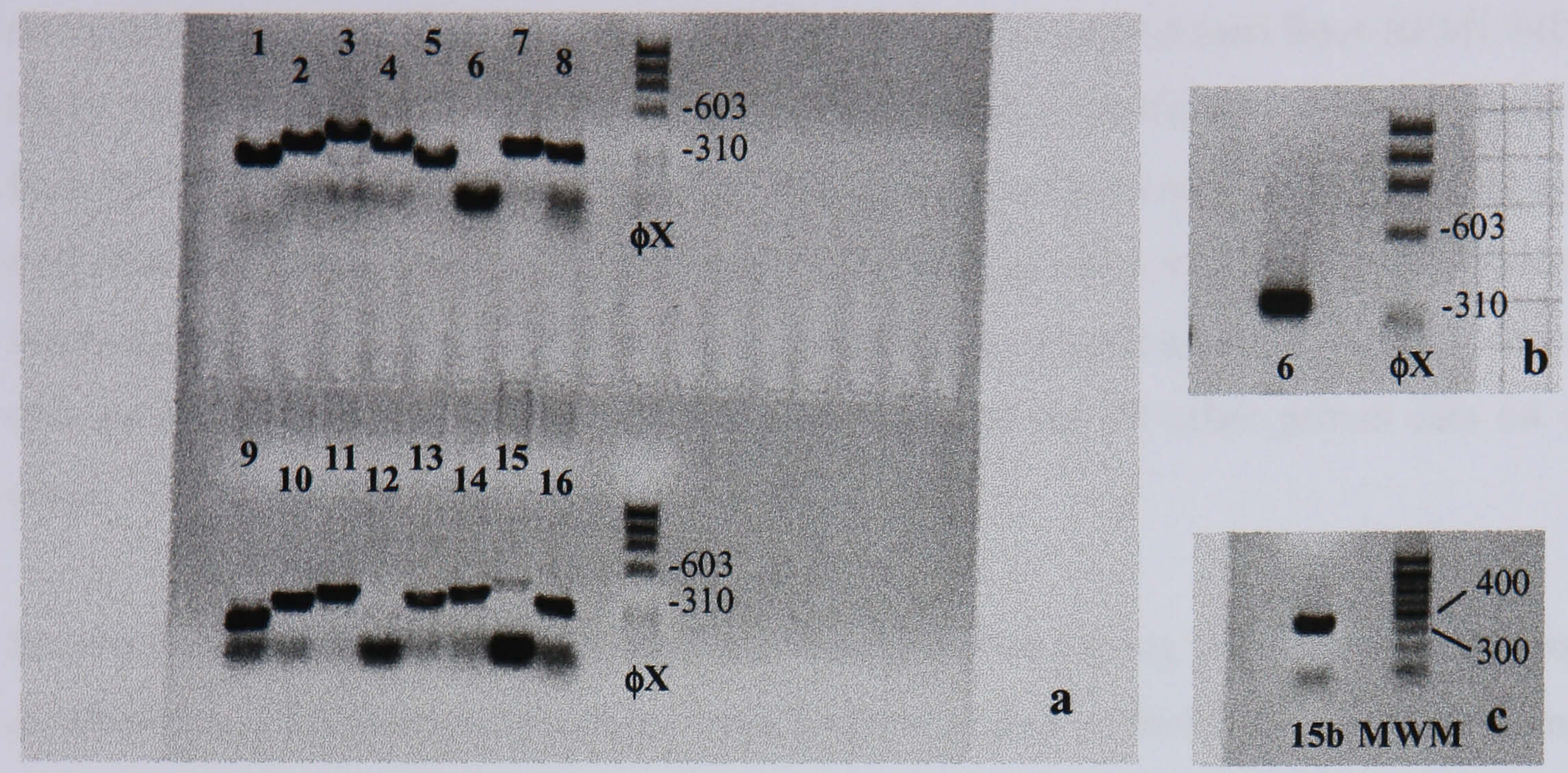


Fig 3.14 Amplification of normal genomic DNA with primers flanking exons of *GluR-6*. (a) for exons 1-16 (b) with a second set of primers for exon 6 (c) with Primers for exon 15b.

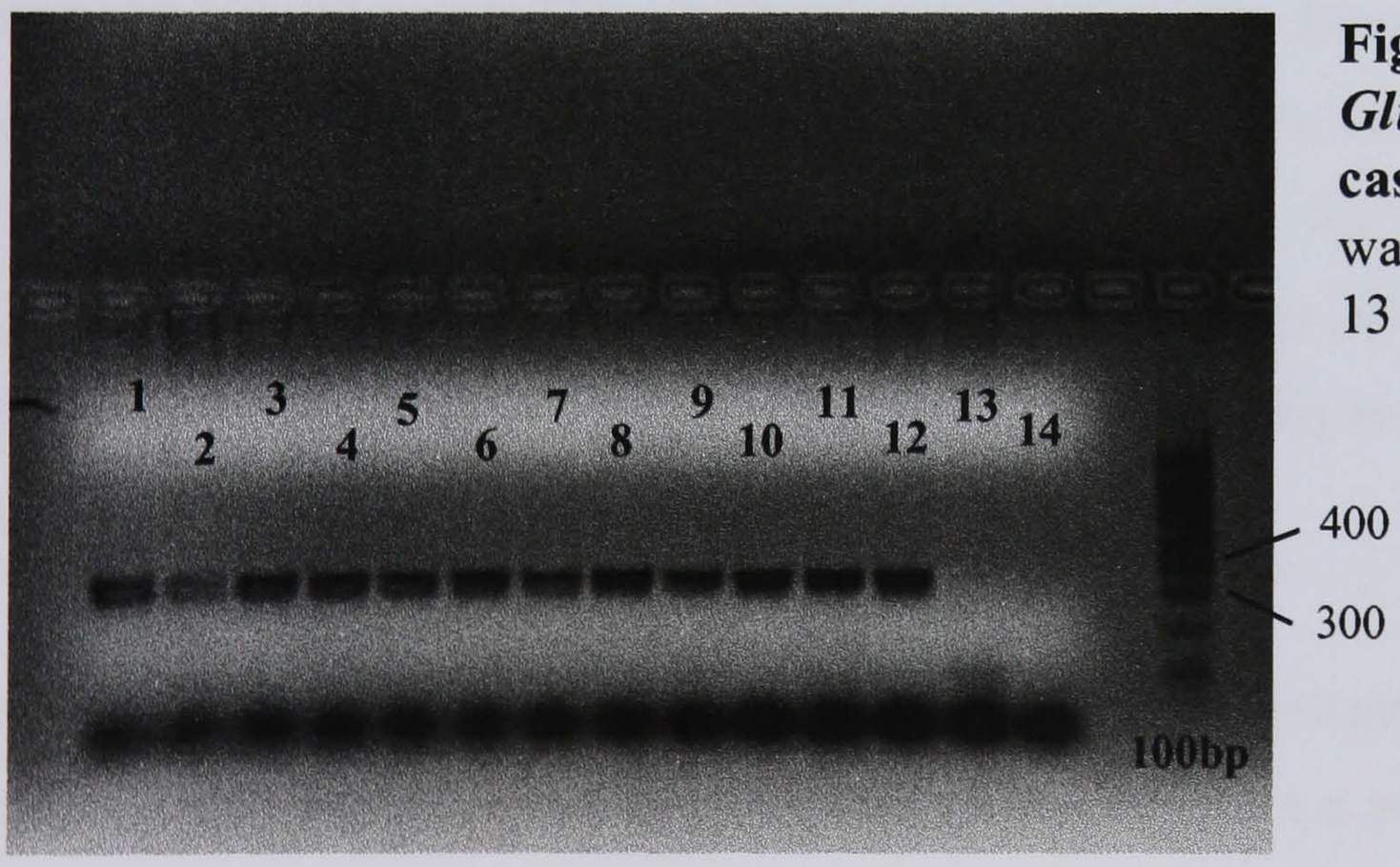


Fig 3.15 Amplification of *GluR-6* exon 6 from 14 cases of ALL. No product was amplified from cases 13 and 14 in this example.

Primers pairs for all the seventeen identified exons of *GluR-6* were used to amplify DNA from the 14 leukaemic cases, with repeat PCR reactions being performed when necessary as detailed in 2.13.2. As an illustrative example the PCR products resulting from amplification of patient and cell line DNA with exon 6 specific primers is shown if **Fig 3.15**. In this case

no visible product resulted from amplification of cases 13 and 14 (cell lines RPMI 8402 and Peer). After repeating the PCR reactions with these cases a satisfactory product was obtained from RPMI 8402 but not from Peer. Despite repeated attempts no visible PCR product was ever obtained from Peer using the exon 6 specific primers raising the possibility that inactivation of the second allele of *GluR-6*, through sub-microscopic deletion, occurred in this instance. A successful PCR reaction was achieved for all other primer sets on all 14 cases.

Forward and reverse sequencing reactions were performed using the cleaned PCR products as templates. GluR-6 exon sequences amplified from cases of leukemia were then compared with corresponding wild type sequences. Sequencing reactions, and where necessary PCR amplification from genomic DNA, were repeated when a mismatch between the amplified leukaemic sample and the wild type sequence was seen in both forward and reverse directions (see **2.13.5** for details of criteria). Sequences obtained from exon 11 consistently included numerous mismatches in both forward and reverse strands. After repeating amplification and sequencing reactions the mismatches were still too frequent to confidently distinguish between mutations and sequencing errors. The intronic regions flanking exon 11 (see appendix 2) contained poly-thymidine repeats that were assumed to have interfered with the sequencing reactions. To avoid sequencing through the repeats, nested forward and reverse primers from just within exon 11 were designed and used to prime sequencing reactions from the original PCR products. As a result of this strategy wild type sequence for exon 11 was obtained from all cases.

The final results of the mutation analysis of *GluR-6* are presented in **Table 3.10**. Wild type sequences were obtained in every case for all exons except 6 and 15. A cytosine to guanine transversion was found at base pair position 113 in exon 6 from patient 1 (**Fig 3.16a and b**). After repeated attempts no PCR product resulted from amplification of DNA with exon 6 primers from case 14 (cell line PEER, as described above). In four patients (2, 5, 8 and 9) a G to A transition was found in exon 15 at position 114. Transition of base pair 114 of exon 15 from G to A would not result in a change in amino acid sequence since both GAG and GAA, encode for a glutamic acid residue. Thus the G to A transition would be silent at the protein

Exon	Patient													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	_	_	_	_	_	_	_	_	_	_	_	_	_	_
2	_	_	_	_	_	_	_	_	_	_	_	_	_	_
3	_	_	_	_	_	_	_	_	_	_	_	_	_	_
4	_	_	_	_	_	_	_	_	_	_	_	_	_	_
5	_	_	_	_	_	_	_	_	_	_	_	_	_	_
6	C→G	_	_	_	_	_	_	_	_	_	_	_	_	NS
7	_	_	_	_	_	_	_	_	_	_	_	_	_	_
8	_	_	_	_	_	_	_	_	_	_	_	_	_	_
9	_	_	_	_	_	_	_	_	_	_	_	_	_	_
10	_	_	_	_	_	_	_	_	_	_	_	_	_	_
11	_	_	_	_	_	_	_	_	_	_	_	_	_	_
12	_	_	_	_	_	_	_	_	_	_	_	_	_	_
13	_	_	_	_	_	_	_	_	_	_	_	_	_	_
14	_	_	_	_	_	_	_	_	_	_	_	_	_	_
15	_	G→A	_	_	G→A	_	_	G→A	G→A	_	_	_	_	_
15b	_	_	_	_	_	_	_	_	_	_	_	_	_	_
16	_	_	_	_	_	_	_	_	_	_	_	_	_	_

Table 3.10 Sequences of exons of *GluR-6* amplified from genomic DNA from cases of ALL with deletions of 6q. Wild type sequence (_), base pair change ($_{X\rightarrow Y}$), PCR failed to result in a visible amplification product (NS).

level, ruling out the possibility that it plays a role in leukaemogenesis. By contrast the C to G transversion observed in exon 6 of patient 1 would result in an amino acid change from Serine (TCG) to tryptophan (TGG) at position N297C of the protein.

To determine whether the C to G change observed in exon 6 of patient 1, resulted from somatic mutation and clonal expansion, DNA from the remission marrow sample from this

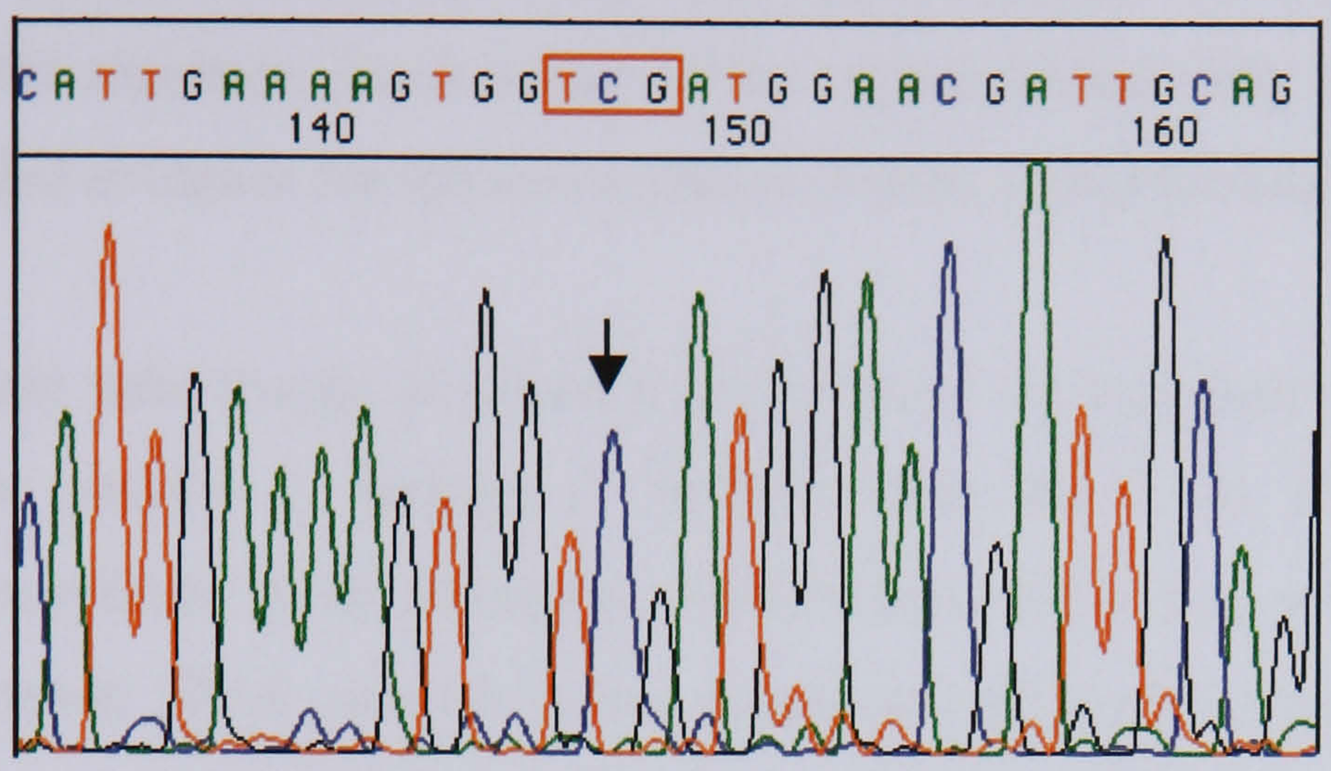


Fig 3.16a Normal Sequence from exon 6 of *GluR6*.

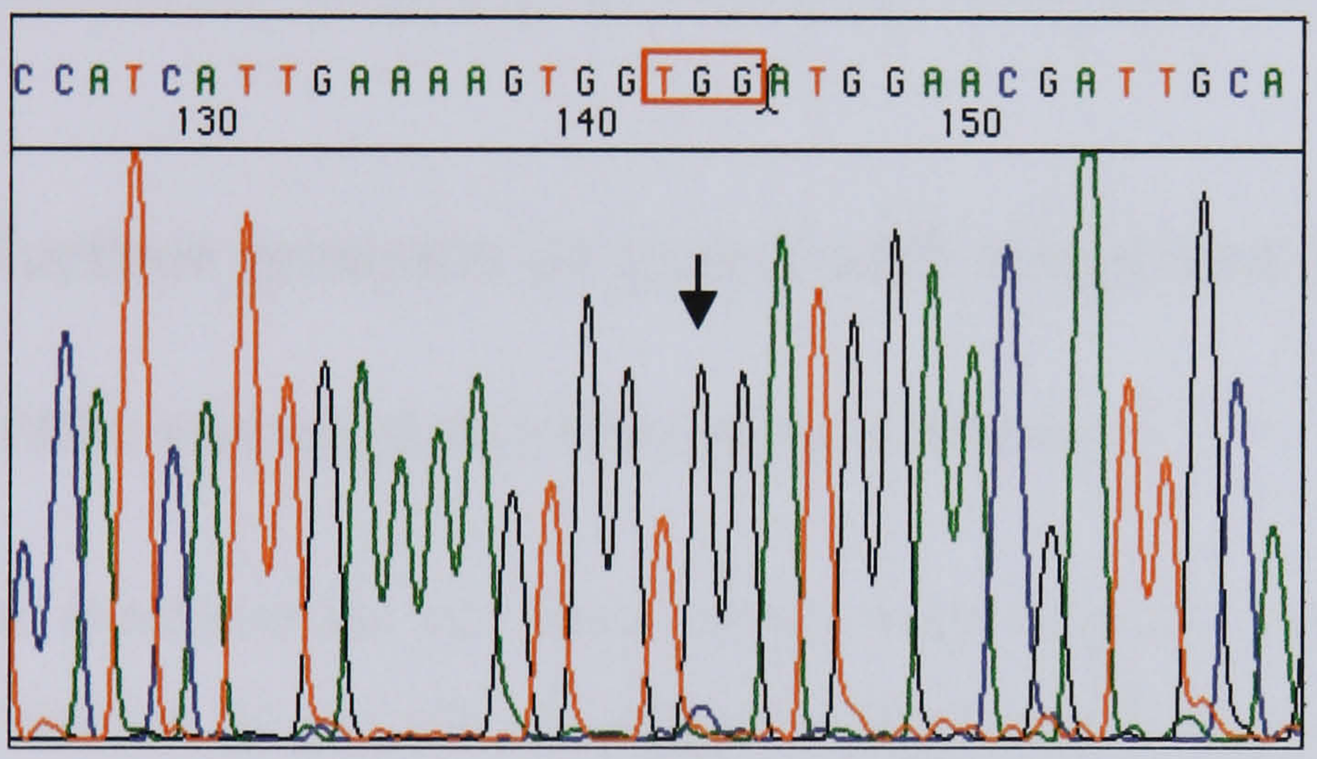


Fig 3.16b *GluR-6* sequence from exon 6 in the presentation sample of case 1. A C to G transversion was present at position 113.

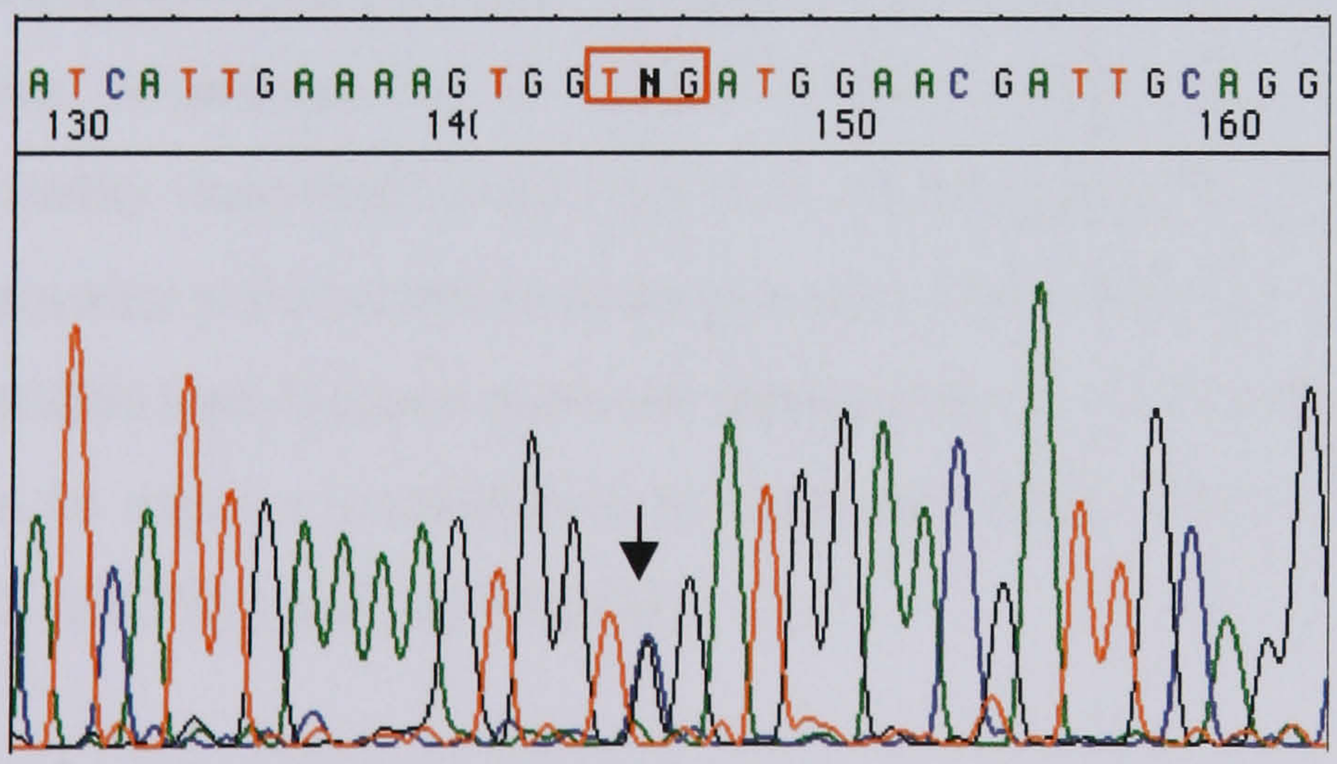


Fig 3.16c *GluR-6* sequence from exon 6 in the remission sample of case 1. Both C and G residues were present at position 113.

patient was also amplified with the exon 6 primers and sequenced. Like the leukaemic sequence, exon 6 from the remission sample displayed evidence for a base pair change at

position 113. The forward sequence gave the same C to G transversion and the reverse sequence a C to N change at the same site, consistent with the presence of both wild type and mutated sequence. Examination of the sequence trace (**Fig 3.16c**) from the remission sample revealed evidence for the co-existence of both C and G nucleotides at position 113.

No base pair change at position 113 of exon six was seen in any of the leukaemic samples except patient 1, making it unlikely that the C to G transversion was a common polymorphism. In an attempt to find evidence for a rare polymorphism at this site a further 20 normal DNA samples were sequenced using the exon 6 primers. All tested normal samples were wild type for exon six, so the possibility remains that patient 1 carried a germ line mutation that conferred a predisposition to develop leukaemia.

3.6 Further analysis of cases with balanced rearrangements of 6q.

3.6.1 FISH analysis of 6q translocation partners.

Partner chromosomes and break points were defined on G-banded analysis for 6 of the translocations of 6q. The identified translocations were t(6;9)(q2?5;q22) (case 26) and t(6;19)(?q21;p13) (case 29) in an ALL, t(6;11)(q13;q23) (case 30), t(6;7)(q2?1;p15) (case 31), t(2;6)(q3?1;q2?3) (case 32) and t(6;8)(q27;p11.2) (case 34) among AML patients. In addition re-analysis of G-banded chromosomes from patient 32 suggested that the abnormality described as add(6q) was a t(3;6)(q26;q25). Chromosome painting with whole chromosome paints confirmed the presence of t(3;6) in this patient (**Fig 3.17a and b**). Of the 6 identified translocation partners, breakpoints in 5 coincided with the location of oncogenes known to make a contribution to leukemia, 19p13 (*E2A*), 11q23 (*MLL*), 7p15 (*HOXA9*), 8p11.2 (*FGFR1*) and 3q26 (*EVI1*).

Rare translocations between *FGFR1* and a gene on 6q27 *(FOP)* had already been characterized (see **1.5**). One of the PACs included in the primary FISH analysis (RP1-167A14) contained the FOP gene sequence. Hybridisation of RP1-167A14 to case 33

Fig 3.17 a. Patient 31: re-analysis of G-banded metaphases suggests that a rearrangement defined as add (6q) is a t(3;6).

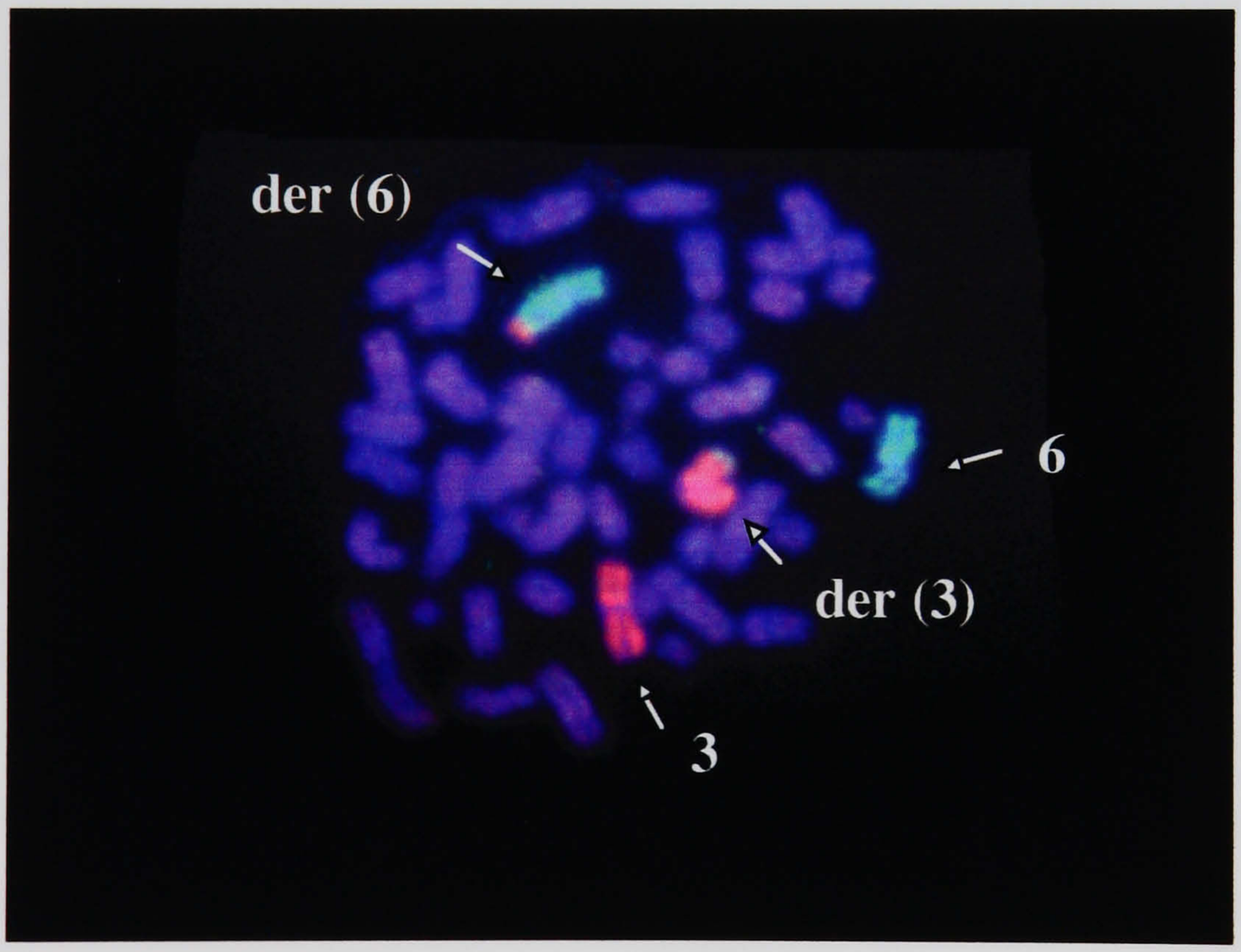


Fig 3.17b. Patient 31: FISH with whole chromosome paints confirms the presence of a t(3;6)(q26;q25).

produced a fluorescent signal of reduced intensity on the derivative chromosome 6 when compared to that seen on the normal homologue. No signal was seen at any other chromosomal location suggesting that a translocation of FOP was accompanied by deletion of 6q material. Further FISH analysis with a YAC containing *FGFR1* (899e2) in combination with RP1-167A14 produced a fusion signal on the derivative chromosome 6 but a signal from 899e2 only on the derivative 8 chromosome (Fig **3.18**). The FISH data therefore suggested that the t(6;8) resulted in fusion between *FOP* and *FGFR1*. Molecular analysis of stored cDNA from this patient confirmed the presence of the chimeric gene (Analysis performed at the Department of Haematology, Hammersmith Hospital) and these data have been recently summarized in a manuscript[126].

FISH analysis with a commercially available dual colour locus specific probe was used to confirm the involvement of MLL in the t(6;11) found in patient 29 (personal communication from the Database). Two recurrent translocations involving MLL and genes on 6q have been cloned. These are the t(6;11)(q27;q23) resulting in production of the *MLL-AF6* oncogene and the t(6;11)(q21;q23) encoding *MLL-AF6q21*(see **1.6.1**) FISH analysis with the primary probes positioned the break point for the MLL translocation included in this study to a region of approximately 5Mb between PACs RP1-67N15 and RP1-235B9 at band q15. Insufficient stored DNA or RNA was available for molecular analysis of this case.

The break point of the t(3;6) was investigated using dual colour FISH with a YAC (14e-e12) which contained sequences of the *EVI1* gene and 6q locus specific probes. A signal from 14e-e6 was seen consistently on the same chromosome arm as the 6q27 specific probe 167A14. No 14e-e12 FISH signal was seen on the der(6) chromosome marked by the 6q centromeric probe RP1-71H19. Since the whole of the *EVI1* gene was contained within 14e-e12 it was concluded that the break point on chromosome 3 was positioned distal to the *EVI1* locus.

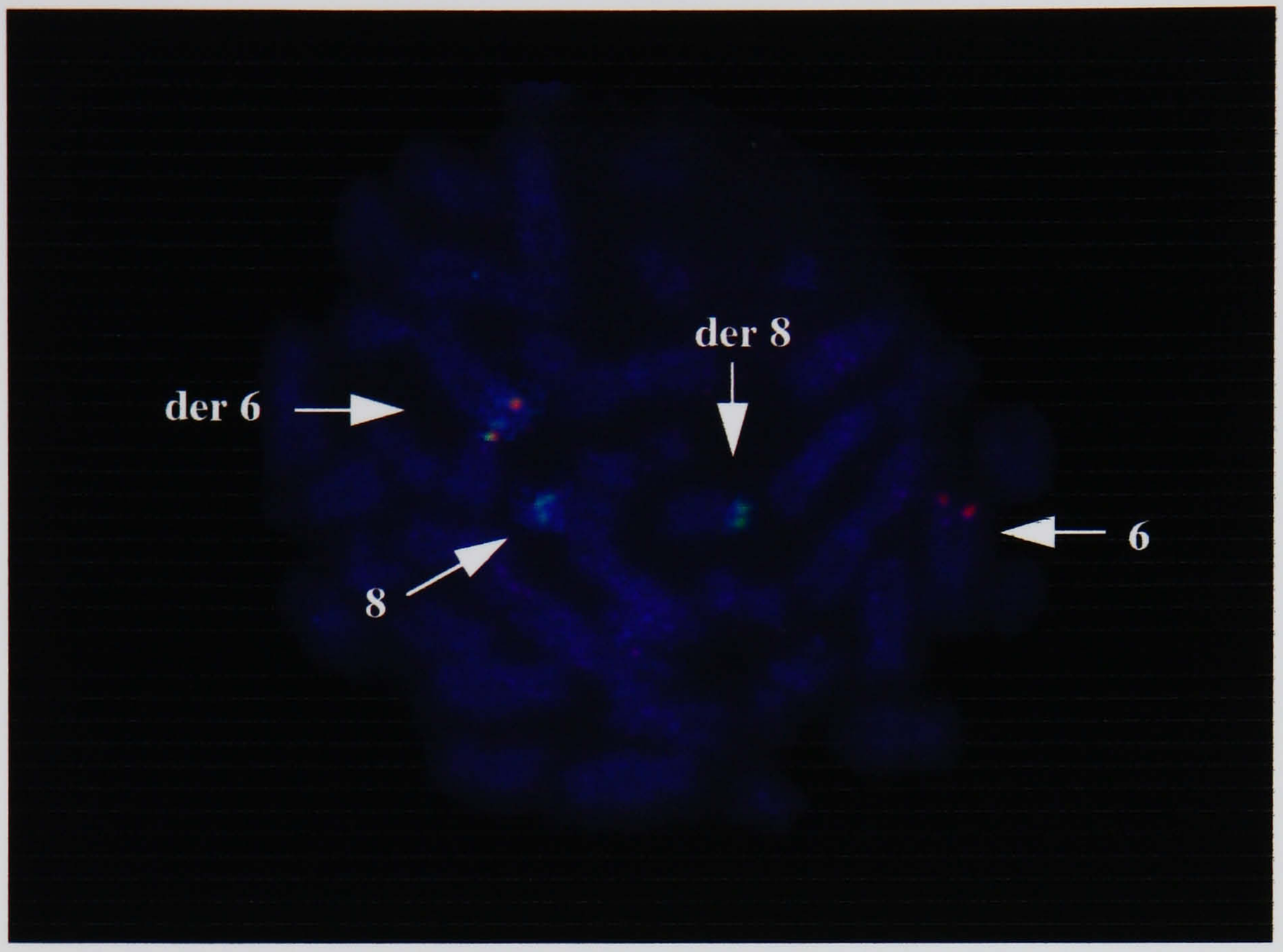


Fig 3.18 FISH analysis of a t(6;8)(q27;p11.2) in patient 34. Green signals; YAC 899e2 (containing the *FGFR1* gene). Red signals;PAC RP1-167A14 (containing the *FOP* gene). 899e2 hybridised to the normal chromosome 8 and as a result of the translocation produced a split signal visible on both the derivative 6 and derivative 8 chromosomes. RP1-167A14 produced a signal on the normal chromosome 6 and a reduced signal on the derivative 6 but no signal on the derivative 8 chromosome. The pattern of signals suggested that fusion between *FGFR1* and *FOP* was in this case accompanied by a deletion of chromosome 6.

3.6.2 Detailed FISH analysis of patients with translocation break points clustered in the 6q22-23 regions.

Analysis with the primary FISH probes revealed 6 translocation/inversion break points in 5 patients that clustered between PACs RP1-293L8 and RP1-172K10. Additional probes positioned between RP1-293L8 and RP1-172K10 were used in FISH experiments to more accurately define the break points in these cases. Patient 29 was also shown to carry a translocation breakpoint within the region, using the new probes directly without completing comprehensive analysis with the primary panel of probes. The probes used with chromosome 6 fpc map positions are presented in **Table 3.11**. The position of the 7 breakpoints with respect to each probe analysed is shown in **Table 3.12**.

Probe	HGMP chromosome 6 map position	Sequenced Fragment Accession Number
RP1-293L8	128350-128500	AL121959
RP1-179E13	129200-129350	AL035603
RP1-28D7	129850-130000	AL109939
RP1-197K23	130500-130600	AL035465
RP1-86D1	130800-130950	AL034349
RP1-150F17	131400-131550	AL589927
RP1-69D17	131850-132000	AL356124
RP1-131F15	134200-134400	AL117378
RP1-55C23	135300-135400	AL032821
RP1-76H23	135800-135900	AL121959
RP1-258L1	137350-137550	AL121943
RP1-12L6	137850-137950	AL353596
RP1-42A6	138400-138550	AL035604
RP1-35M23	139050-139250	AL023284
RP1-199H14	139650-139850	AL357082
RP1-117-D4	140350-140500	AL023580
RP1-171N11	141100-141300	AL031433
RP1-172K10	142850-143000	AL022477

Table 3.11 PACs used for secondary FISH analysis of translocation breakpoints falling between 293L8 and 172K10. Left and right hand HGMP chromosome 6 map positions are given for each clone.

Probe	Cases Number						
	25	26	27	28	29	32a	32b
RP1-293L8	C	C				C	C
RP1-179E13		C	C			T	C
RP1-28D7						T	C
RP1-197K23						T	C
RP1-86D1					C	T	C
RP1-150F17						T	C
RP1-69D17	C				C	T	C
RP1-131F15			C		T		
RP1-55C23			C				
RP1-76H23	T	C	T	C		T	C
RP1-258L1			T			T	C
RP1-12L6				C	T	T	C
RP1-42A6				T		T	C
RP1-35M23				T		T	C
RP1-199H14				T		T	C
RP1-117-D4				T		T	T
RP1-171N11	T		T	T		T	T
RP1-172K10	T	T		T		T	T

Table 3.12 Secondary FISH analysis of cases of AML/ALL with translocation/inversion breakpoints falling between 293L8 and 172K10. Abbreviations are follows: **C**: probe positioned on the centromeric side of the translocation break point. **T**: probe positioned on the telomeric side of the break point.

In two cases, numbers 25 and 26, the extent of analysis was limited by the availability of suitable cytogenetic material and break points were mapped only to within relatively wide regions. Of the remaining five rearrangements, four were positioned to within 1 Mb intervals and one (patient 29) to within approximately 2.5 Mb. The five breakpoints, mapped in detail, were shown to occur at different positions on 6q. The interval containing the break point from case 25, overlapped with both those for cases 29 and 27. Similarly, the wider region defining a breakpoint from patient 26 contained more precisely mapped translocation (patient 28) and inversion (patient 32) breakpoints. The translocation in case 32 had the most

centromeric breakpoint and was separated from the co-existent inversion by not less than 11 Mb.

3.6.3 Gene content of the 6q22-23 break point cluster region.

The probes used for secondary analysis of the clustered breakpoints were then positioned with respect to sequenced fragments using the Sanger Center ACEDB and ENSEMBL human genome browsers. Apart from two unsequenced gaps of approximately 50Kb, the region between RP1-293L8 and RP1-172K10 contained 157 sequenced fragments forming a complete 14.8 Mb contig. The region contained a large number of genescan predicted genes and NCBI curated transcripts, 91 of which were classified as genes by ENSEMBL or EMBO. A known or predicted function had been assigned to 60 of the recognized genes. Names and details of transcript size, number of exons, genomic size, ENSEMBL I.Ds and functions of the genes are presented in **Tables 3A.3** and **3A.4** (appendix 3). The position of the genes with respect to the PACs used for FISH analysis and the translocation/inversion breakpoint regions for each patient is presented in **Fig 3.19**. The gene content of the five breakpoint regions, mapped in detail, will be briefly described in a centromeric to telomeric order.

Patient 32 (translocation). The most proximal of the 7 break points falling between RCPI1-293L8 and RCPI1-179E13 contained three known/predicted genes and three genes of unknown function. Two genes *HEY2* and *Q9NQES* were similar to genes expressed at specific stages of development in Drosophila. *Q9NQE9* coded for a gene with homology to protein kinase C inhibitors. Protein kinase C inhibitors modulate activity of cyclin dependent kinases that in turn control cell cycle progression through phosphorylation of the Rb protein. Recently protein kinase C inhibitors have been demonstrated to have anti tumourogenic effects[269].

Patient 29. The 2.5 Mb region encompassing the t(6;19) translocation break point contained 5 genes of unknown and 10 genes of known or postulated function. Most centromeric of these and falling only partly within the region was *LAMA 2*, a large (9.5 kb) gene, mutations of which are the cause of merosin deficient muscular dystrophy. *EPB41L2* encodes a member

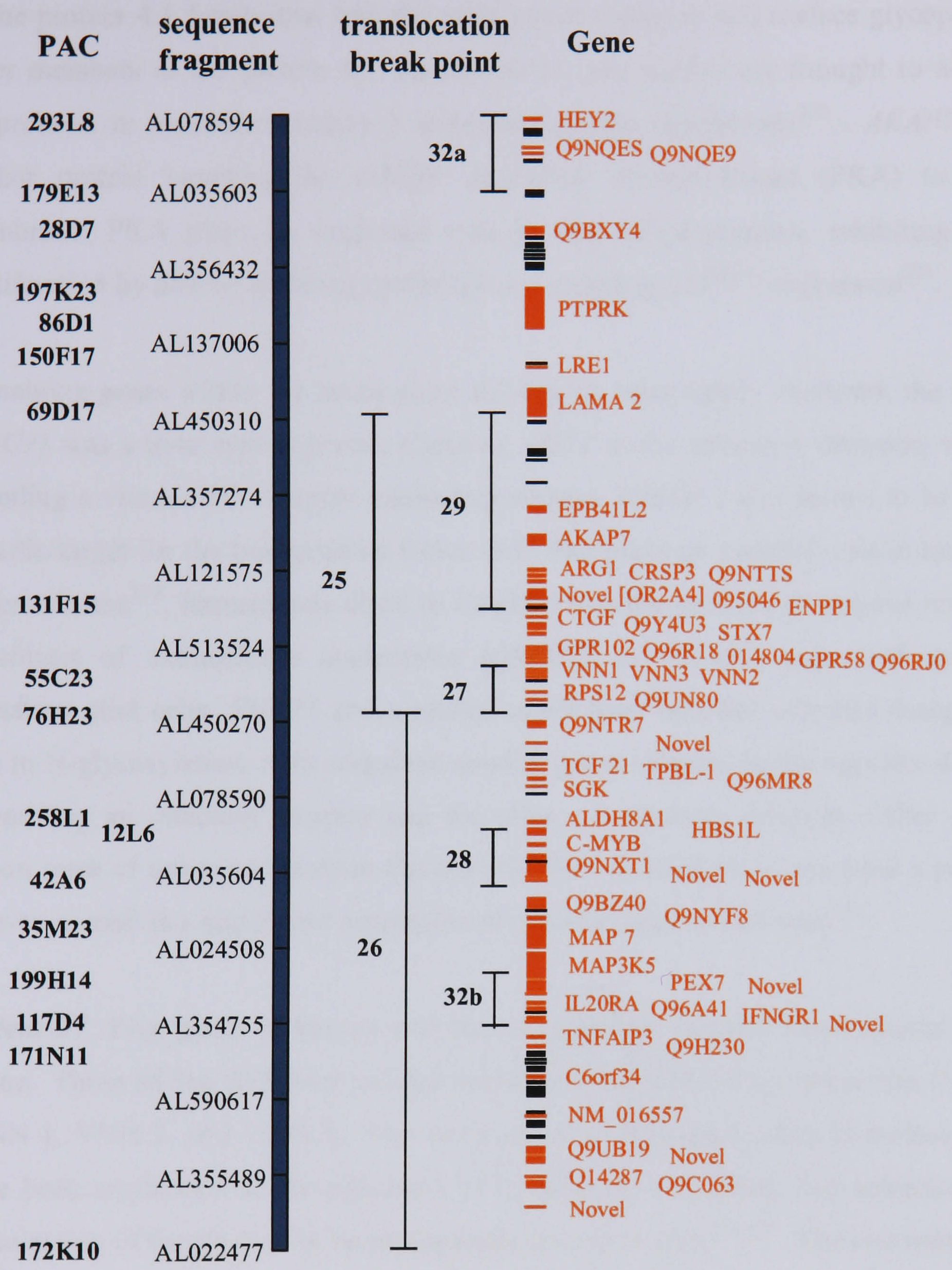


Fig 3.19 Gene content of the 6q22-23 break point cluster region derived from FISH analysis of cases of acute leukaemia with cytogenetic deletion of chromosome 6.
Sequenced fragment accession numbers are given at 1 Mb intervals. The position of genes of known or predicted function are marked in red with their ENSEMBL IDs. Genes of unknown function are marked in black without corresponding IDs.

of the protein 4.1 family that link the actin cytoskeleton to cell surface glycoproteins. Two other members of the protein 4.1 family, *merlin* and *DAL-1* are thought to act as tumour suppressors in neurofibromatosis 2 and meningiomas respectively[270]. *AKAP7* encodes an anchor protein targeting the c-AMP dependent protein kinase (PKA) to the plasma membrane. PKA plays an important role in immunosuppression, inhibiting lymphocyte proliferation by down-regulating cyclin D3 and inducing $p27^{(kip1)}$ expression[271].

Remaining genes within the break point sub-region were tightly clustered, the first of these (*ARG1)* was a liver type arginase. Close to *ARG1* in the telomeric direction was *CRISP 3* encoding a vitamin D3 receptor interacting protein. *CRISP 3* was shown to be a pre-B cell specific target for the transcription factor Oct2 that plays an essential role in terminal B cell differentiation[272]. Immediately distal to *CRISP 3* was the gene for an enzyme involved in the hydrolysis of extracellular nucleotides (*Q9NTTS* or *PDNP3*) expressed specifically in immature glial cells. *ENPP1* and a related novel gene encoded enzymes thought to play a role in N-glycosylation, both contained smaller genes oriented in the opposite direction, one resembling an olfactory receptor and the other of unknown function. Other genes in the region were of unknown function but one (095046 or *MGEA6/11*) encoded a protein that is over-expressed in a significant proportion of meningiomas and gliomas[273].

Patient 27. Five genes of known and two of unknown function were located in this sub-region. Three of the five were related vascular non-inflammatory molecule (Vanin) genes (VNN 1, VNN 2, and VNN 3). The vanin genes are thought to have hydrolase activity and have been implicated in the processes of lymphocyte migration, cell adhesion and in the colonization of the thymus by haematopoietic precursor cells[274,275]. The two remaining genes of know function, RPS12 and Q9UN80 code for an S40 ribosomal protein and a Line 1 reverse transcriptase, respectively.

Patient 28. This translocation break point fell between PACs RCPI1-12L6 and RCPI1-42A6, a region encompassing all or part of six genes only one of which was of unknown function. The most proximal gene (*HBS1L*) positioned partly on 199H14, coded for a member of the elongation factor 1 alpha family. The yeast homologue of HBS1L (HBS1) is a G protein that

interacts with the ribosome and regulates steps in the process of protein synthesis[276]. The entire sequence of the known proto-oncogene *C-MYB* was positioned within the region. *C-MYB* is expressed at high levels in immature haematopoietic cells where it plays important roles in growth regulation and differentiation[277]. Activated truncated forms of *C-MYB* have been isolated from chicken leukaemia viruses and also from a cell line established from a patient with T-cell blast crisis of CML and a del (6q)[278]. Overexpression of normal C-MYB has also been described in haematopoietic malignancies.

A large (5.267 kb) gene of uncertain function, *Q9NXT1*, was positioned within the sub-region and distal to *C-MYB*. *Q9NXT1* encoded several known functional domains including one homologous to the Rec A bacterial recombination protein, suggestive of a role in DNA repair. A smaller gene of unknown function was positioned within *Q9NXT1* and a further two genes coding for glyceraldehyde 3 phosphate dehydrogenases also fell within this translocation break point region.

Patient 32 (inversion). The most distal of the break points mapped in detail contained seven genes of known and one of unknown function. The most proximal, *MAP3K5*, was positioned largely outside the region and therefore was unlikely to be interrupted by the translocation. However, *MAP3K5* encodes a MAP kinase kinase kinase that plays an important role in the regulation of apoptosis. Cytotoxic stresses such as exposure to TNF, FAS or reactive oxygen species activate MAP3K5, which in turn activates JNK and p38 pathways. *MAP3K5* -/- cells were shown to be resistant to TNF and H_2O_2 induced apoptosis[279].

Of the genes positioned unambiguously within the break point region, one coded for a peroxisomal targeting signal receptor (*PEX 7*) and one for a novel amino acid transporter protein. Also within the region were three class II cytokine receptors, the interleukin 20 receptor alpha (IL20RA), the interleukin 22 binding protein (Q96A41 or IL-22BP) and the interferon-gamma receptor alpha chain precursor (IFNGR1). IL20RA transduces growth promoting signals through STAT3 in keratinocytes and is upregulated in psoriatic skin[280]. IL-22BP apparently acts as an agonist, blocking binding of IL-22 and subsequent STAT activation through the functional receptor. Expression of IL-22BP was reported to be highest

in breast, lung and colon. The third cytokine receptor IFNGR1 is secreted by natural killer cells and T-lymphocytes, dominant negative mutations within the gene confer susceptibility to mycobacterial infections[281]. Of the two remaining genes in the sub-region one was of unknown function and the other coded for a novel protein kinase C binding protein.

3.6.4 Detailed FISH analysis of a complex duplicated translocation.

A translocation between 6q and an unknown partner chromosome, with a break point between PACs RP1-65A9 and RP1-167A14 at band q27, was detected by FISH analysis using the primary probes (see section **3.2.3** ***patient 22***). A complex pattern of signals, consistent with duplication of one of the translocation products accompanied by deletion, resulting from unbalanced translocation of the second homologue of 6q, was observed. Sequential FISH experiments with probes mapped to between RP1-65A9 and RP1-167A14 (**Table 3.13**) positioned the break point of the translocation to between PACs RP1-249F5 and RP4-655C5 (**Fig 3.20**). The two PACs positioned on either side of the translocation break point both contained part of the *Insulin-like growth factor II receptor/Mannose-6-phosphate receptor* gene (*IGF2-R/M6PR*). According to left and right hand fpc values, a gap existed between the two clones (right hand RP1-249F5 = 177215 – left hand RP1-655C5 = 177394). However, the NCBI database positioned the two clones contiguous to each other. RP1-249F5 was fully sequenced but RP1-655C5 is still in several unordered pieces. BLAST search with the 48 individual exons of *IGF2R* positioned exons 1-4 on RP1-249F5, and the following exons on RP4-655C5: 5, 8-13, 16-19, 20-21, 31-33, 36-39, and 40-46. Based on the FISH data the most likely position for the translocation break point in patient 22 was therefore between exons 4 and 5 of the *IGF2-R*. All the *IGF2-R* exons from 5 to 48 could be positioned on a PAC (RP1-288H12) that occupied the same genomic region as RP1-655C5 but which had been fully sequenced.

3.6.5 Southern blot analysis of the *IGF2-R* (patient 22).

A Southern blot was made from 3 remission bone marrow DNA samples and from DNA from patient 22 leukaemic bone marrow taken on two different occasions, including the date on which the cytogenetic sample used for FISH analysis was taken. BamH1 and EcoR1

digests of each sample were used to prepare the Southern blot (**Fig 3.21**). The blot was then probed sequentially using IGF2-R SB probes 1-6 (see **2.13** for details of preparation and **Fig 3.22** for image of the probes after gel electrophoresis).

Probe	Chromosome 6 fpc values	Cytogenetic band	Marker	Gene
RP1-65A9	170802-170937	6q27	D6S305	
RP1-56L9	176761-176977	6q27		
RP1-249F5	177059-177215	6q27		*MAS1/IGF2-R*
RP4-655C5	177394-177638	6q27		*IGF2-R*
RP1-247I20	177569-177748	6q27		
RP1-102N2	178212-178336	6q27		
RP1-167-A14	178304-178414	6q27		*FOP*

Table 3.13 PACS used for secondary FISH analysis of the translocation of 6q seen in patient 22. Showing right and left hand ACEDB chromosome 6 map positions for each clone and corresponding band assignments, genetic markers and genes.

The position of probes with respect to *IGF2-R* exons and the sequences of PACs RP1-249F5 (sequence accession # AL035691) and RP1-288H12 (sequence accession # AL353625) are presented in **Table 4.A.1** (Appendix 4).The two PAC sequences were analysed using DNASTAR software to determine the position of BamH1 and EcoR1 restriction enzyme sites. The position of restriction enzyme sites and calculated expected fragment sizes resulting from digestion of normal genomic sequence are also presented in **Table 4.A.1**. The position of *IGF2-R* exons, restriction enzyme digest fragments and the five probes, with respect to clones RP1-249F5 and RP1-288H12 (and RP4-655C5), are also represented in **Fig 3.23**. Restriction enzyme fragments that do not overlap with one of the exons are also highlighted in **Fig 3.23**.

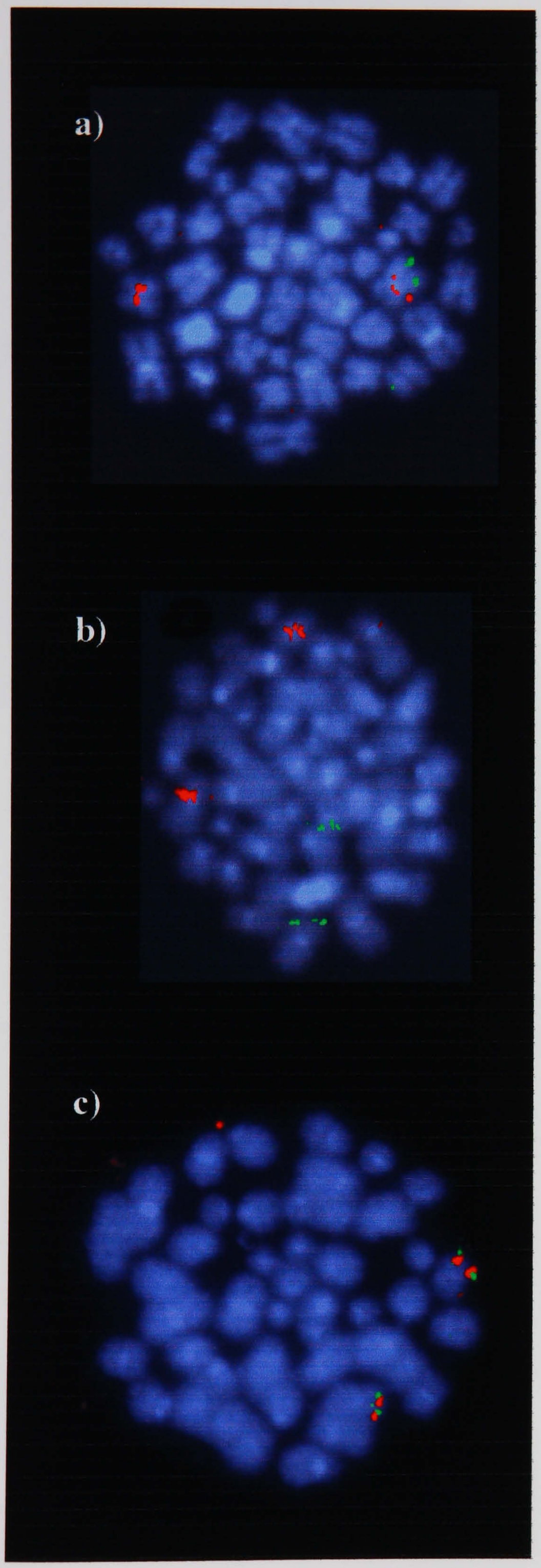


Fig 3.20 FISH analysis of Complex rearrangements of chromosome 6 found in patient 22.

a) Red signals; RP1-71H19 (marking the 6q centromeric region). Green signal; RP1 249F5 (containing exons 1-4 of the *IGF2-R* gene). A single green signal is seen on a chromosome marked by the red probe, and consistent with the presence of deletion, absent from a second smaller chromosome hybridising to the 6q centromeric probe.

b) Red signals; RP1-71H19. Green signals; RP1-655C5 (containing exons 5-48 of the *IGF2-R*). Two green signals are seen, neither hybridising to the chromosome marked by the 6q centromeric probe.

c) Red signal; RP1-167A14 (marking the 6qtelomeric region). Green signals; RP1-655C5. Red and green signals co-localiseon two marker chromosomes.

Taken together the pattern of FISH signals is consistent with a scenario in which deletion of one homologue of 6q was accompaniedby a translocation that interrupted the *IGF2-R*. Duplication of the translocation product containing the distal part of 6qhas taken place and since the two chromosomes marked by 6q telomeric probes are of different sizes, further rearrangement of at least one of the markers had occured.

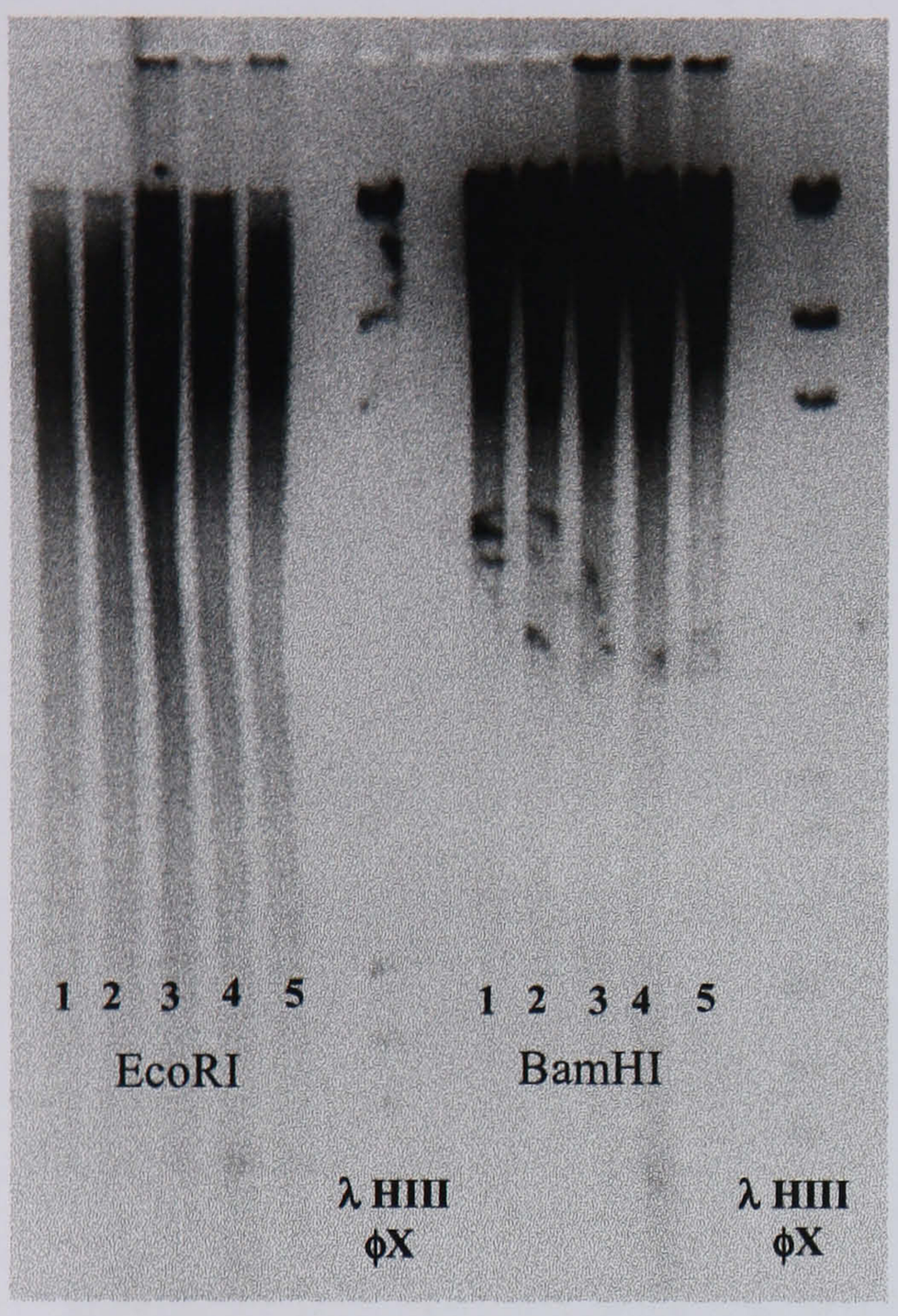
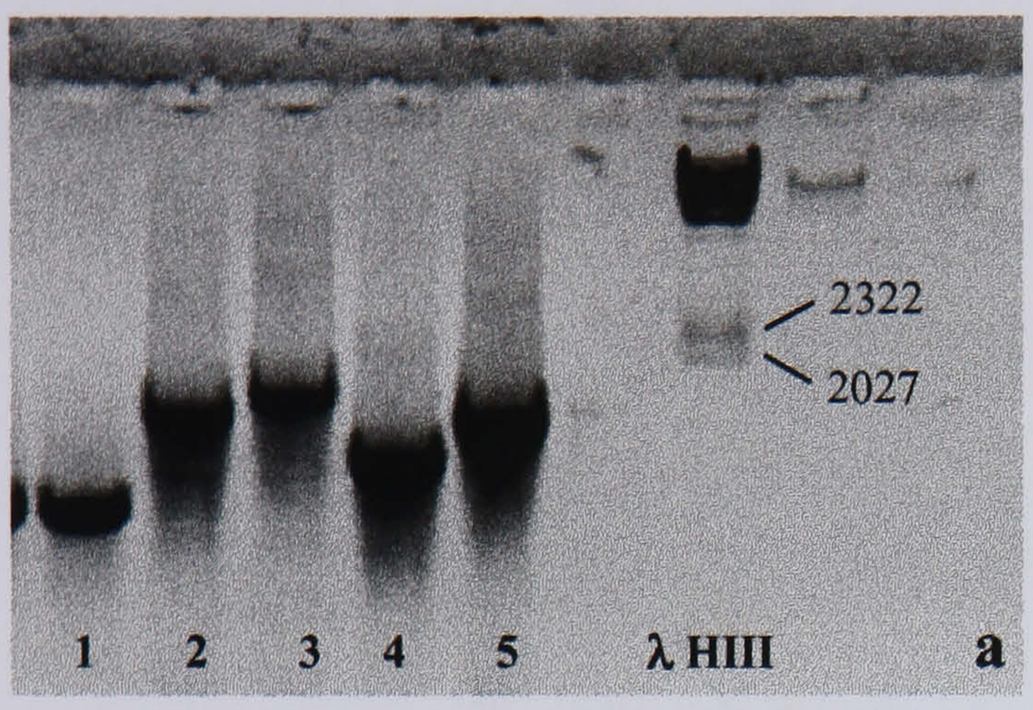


Fig 3.21 Patient 22 and reference DNA digested with restriction enzymes and run on an agarose gel for Southern blot preparation. (1) patient 22 sample date 21.9.98 when 50% of cells carried a translocation of 6q according to FISH analysis, (2) patient 22 sample date 13.2.98 when the patient was reported to carry a cytogenetically detectable deletion of 6q though no FISH analysis had been performed, (3-5) lymphoma patients with no evidence for 6q translocation.



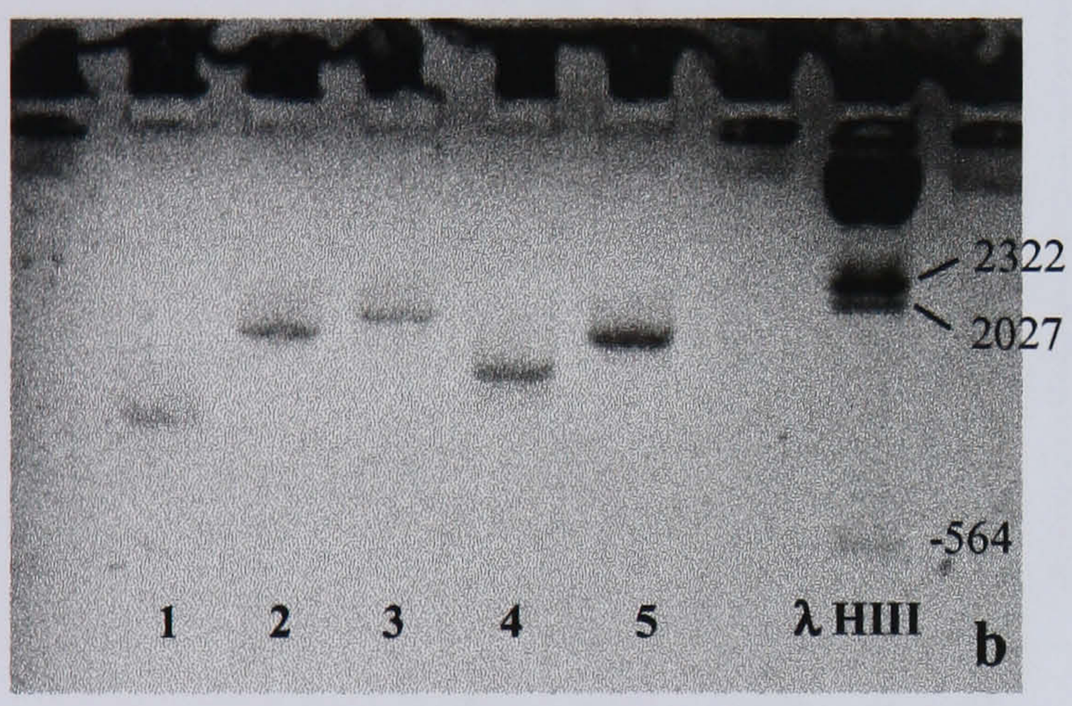


Fig 3.22 Probes for Southern blot analysis amplified from the *IGF2-R* cDNA. Probes 1-5 before (a) and after (b) excising from gel and cleaning.

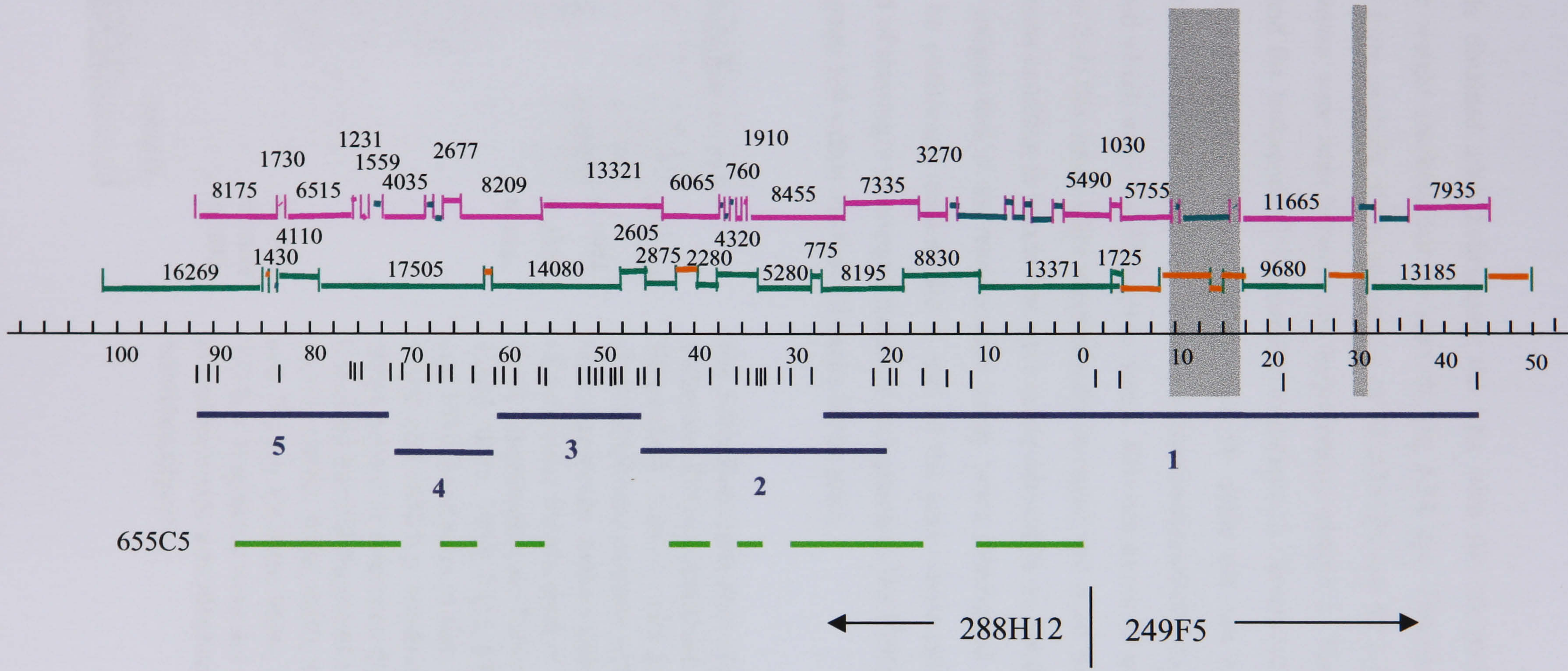


Fig 3.23 Southern blot analysis of the *IGF2-R*. Relative position of the 5 probes with respect to the sequence of PACs RP1-288H12, RP1-249F5 and RP4-655C5, exons of *IGF2R* and BamH1 and EcoR1 fragments. Green bars indicate BamH1 fragments which contain exon sequence, orange bars BamH1 fragments containing only intronic sequences. Pink bars indicate EcoR1 fragments containing exon sequences and blue bars EcoR1 fragments without exon sequence. The grey boxes cover areas where neither BamH1 or EcoR1 fragments contain exon sequences.

The bands obtained after hybridizing the blot with the six probes and the position of molecular weight markers can be seen in **Fig 3.24 a-e**. Though some of the fragments predicted from analysis of the sequences of RP1-249F5 and RP1-288H12 were not visible, no differences were seen between the hybridisation pattern in the remission bone marrow samples and the leukaemic DNA samples in patient 22. Several of the predicted restriction fragments positioned between exons 1 and 10 either did not overlap with an exon or overlapped with only a single exon so would have been predicted to give either no signal or a weak signal which would be difficult to detect. Between exons 12 and 48 (covered by IGF2R SB probes 2-4) the exons were more closely arranged and most fragments overlapped with several exons resulting in bands that were easily detectable on Southern blot analysis. It was therefore judged that if the translocation break point interrupted the *IGF2-R* it was most likely to be positioned towards the 5' end of the gene where introns were larger and the likelihood of missing a rearranged fragment was greatest. The FISH data also suggested that the breakpoint fell within the first 10 exons of the gene.

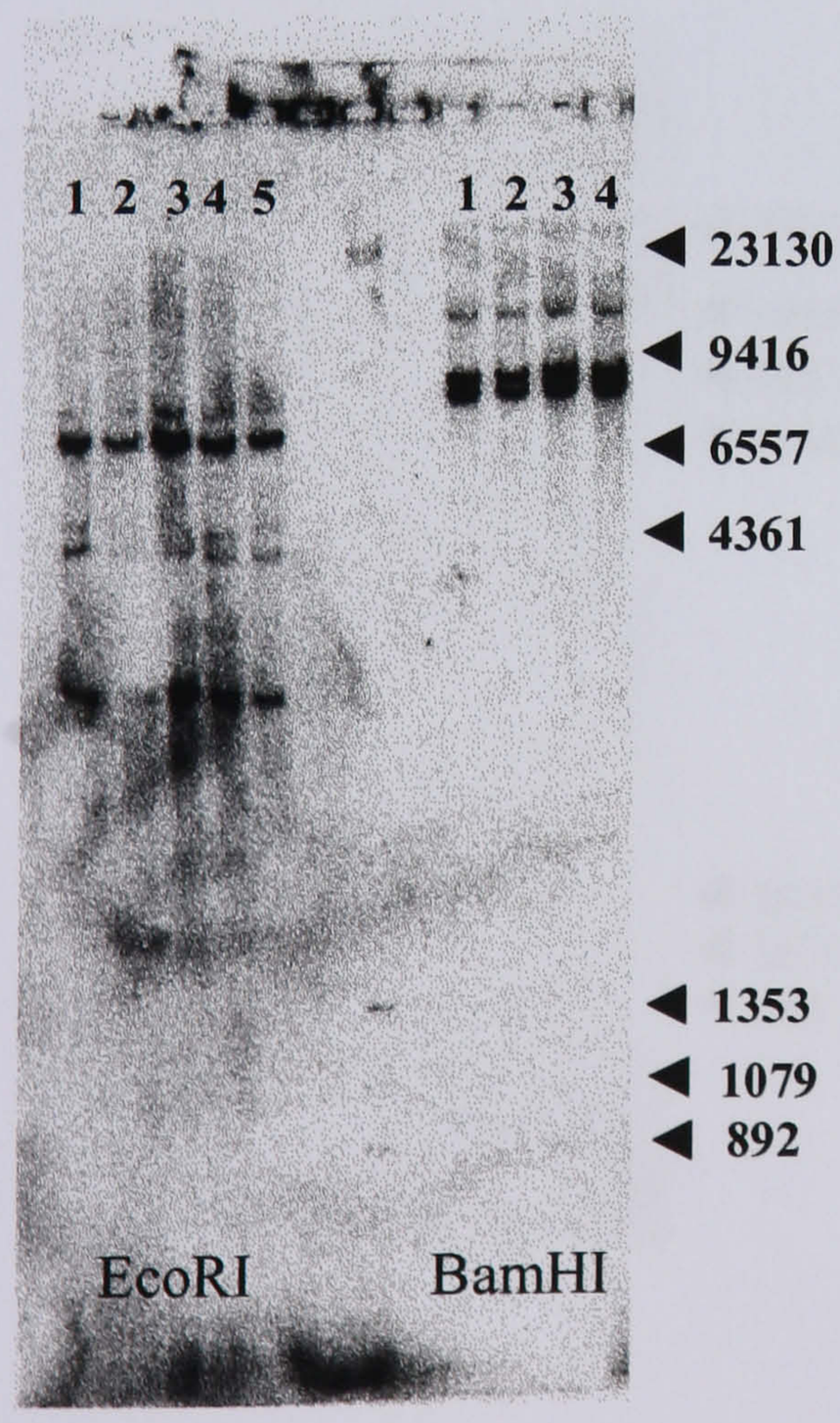


Fig 3.24a Southern blot of patient 22 and reference DNA hybridised with IGF2-R SB probe 1. Lanes (1 and 2) patient DNA, (3-5) lymphoma patients without 6q rearrangements. Arrows indicate the position of molecular weight marker bands. Expected EcoR1 fragments (see Table 3.17) were; 11665, 8455, 7935, 7335, 5755, 5490,3270 and 1030, base pairs in size. Of these the 11665 and 1030 b.p. bands could not be seen in either patient or reference digests.
Expected BamHI fragment sizes were; 13371, 13185, 9680, 8830, 8195, 5280, 1725 and 775 b.p. Of these 9680, 5280, 1725 and 775 b.p. fragments were not seen. The pattern of visible bands was identical in patient 22 and reference digests.

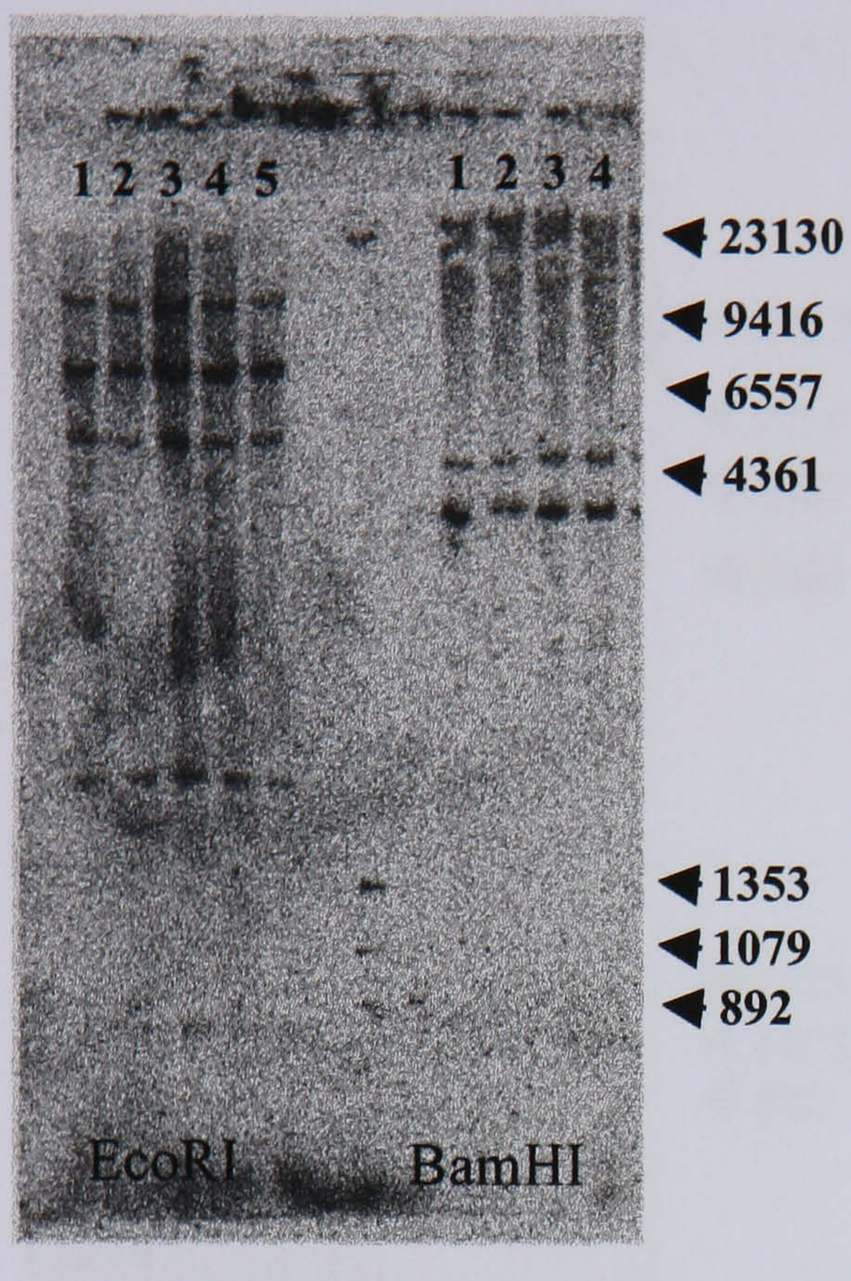


Fig 3.24b Southern blot of patient 22 and reference DNA hybridised with IGF2-R SB probe 2. Expected EcoRI fragment sizes were; 13321, 8455, 7335, 6065, 1910 and 760 b.p., of these, either the 8455 or 7335 b.p band was not present. The expected BamH1 digest fragment sizes were 8195, 5280, 4320 2875, 2280, 2605 and 775 b.p. Of these only the 5280 and 4329 b.p. bands were visible. Restriction digest fragment sizes appeared identical in patient and reference samples.

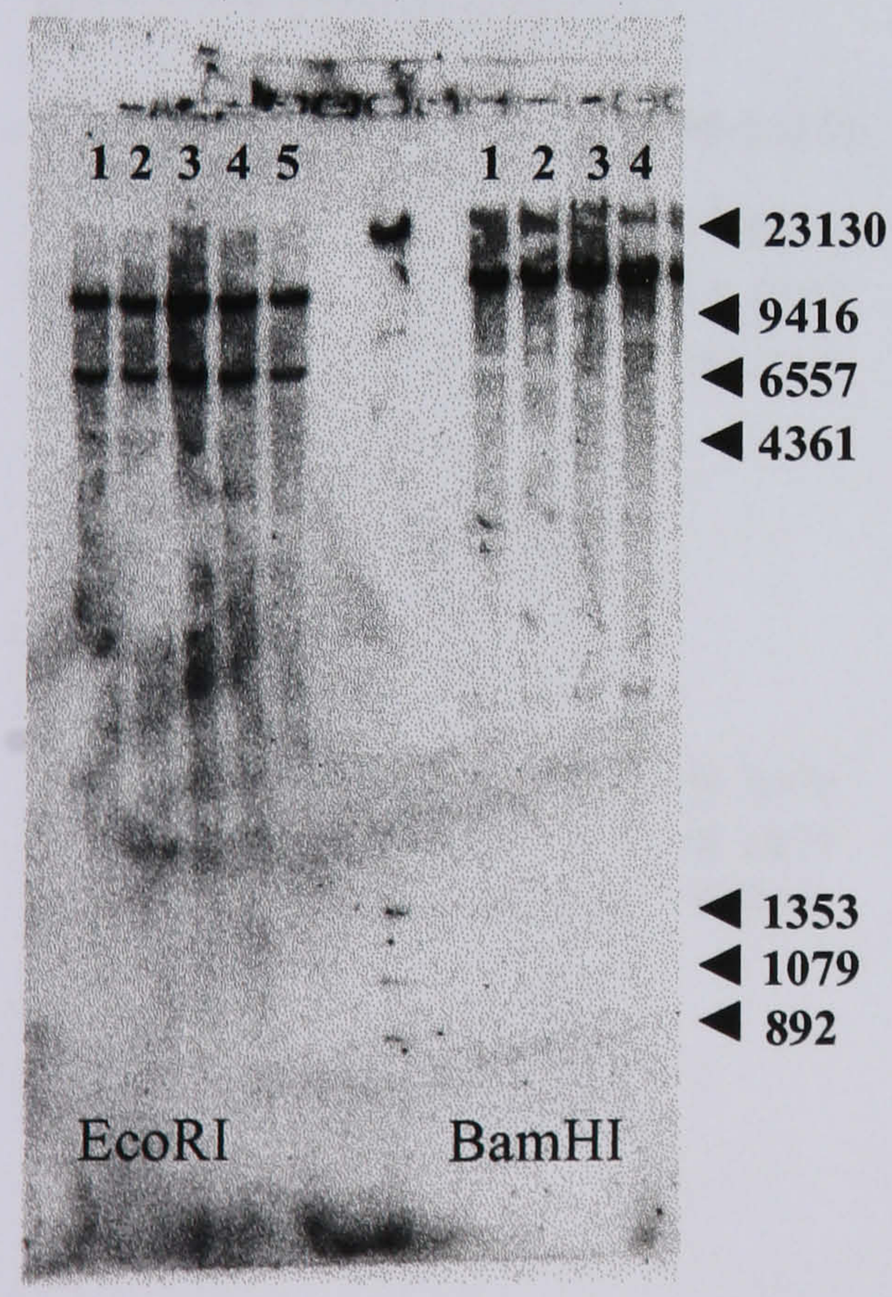


Fig 3.24c Southern blot of patient 22 and reference DNA hybridised with IGF2-R SB probe 3. Expected EcoRI digest fragment sizes were; 13321 and 8209 b.p. Expected BamHI fragment sizes were 2605 and 14080 b.p. Bands corresponding to the expected fragment sizes were seen in patient and reference samples digested with both EcoRI and BamHI.

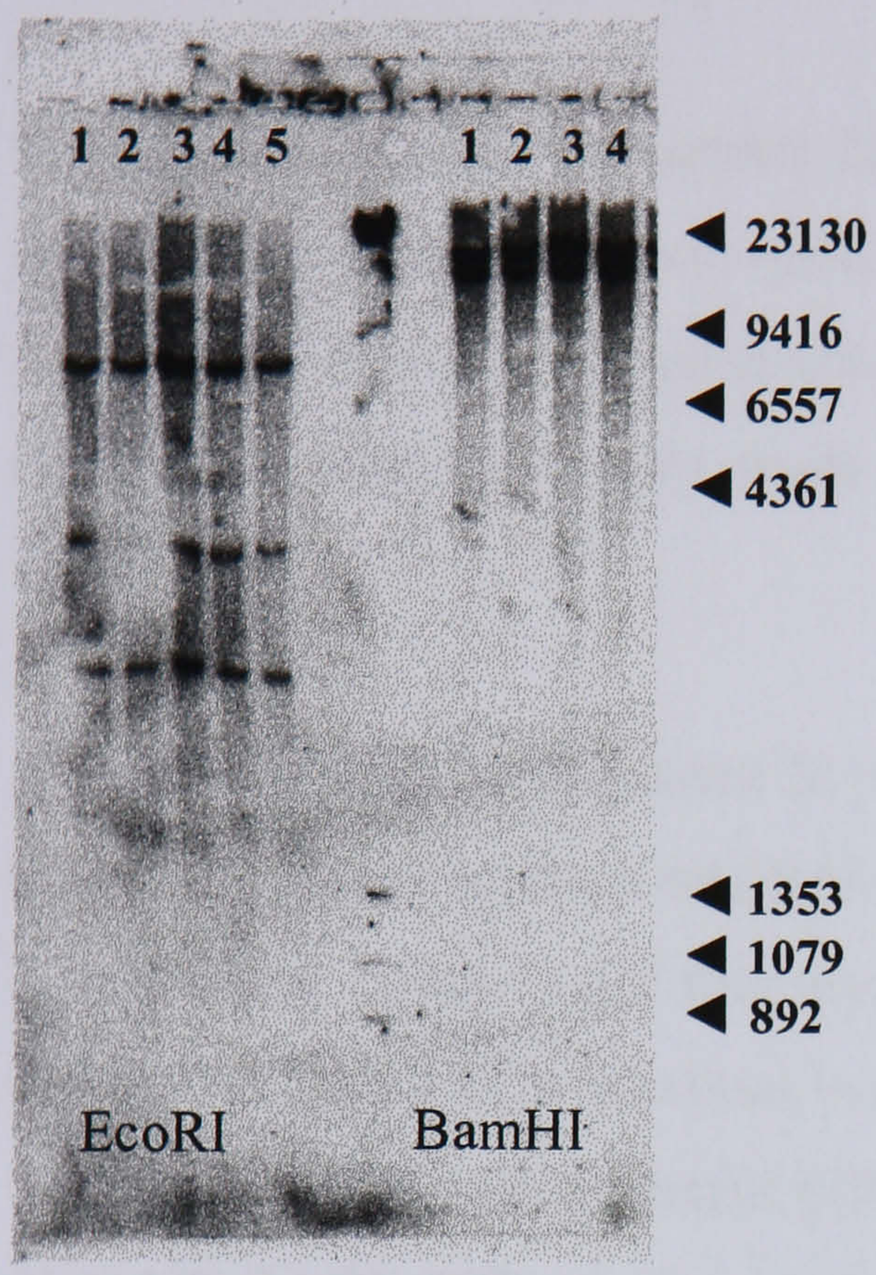


Fig 3.24d Southern blot of patient 22 and reference DNA hybridised with IGF2-R SB probe 4. Expected EcoRI digest fragment sizes were; 8209, 4035 and 2677 b.p. Expected BamHI fragment sizes were 17505 and 14080 b.p. Bands corresponding to the expected fragment sizes were seen in patient and reference samples digested with both EcoRI and BamHI.

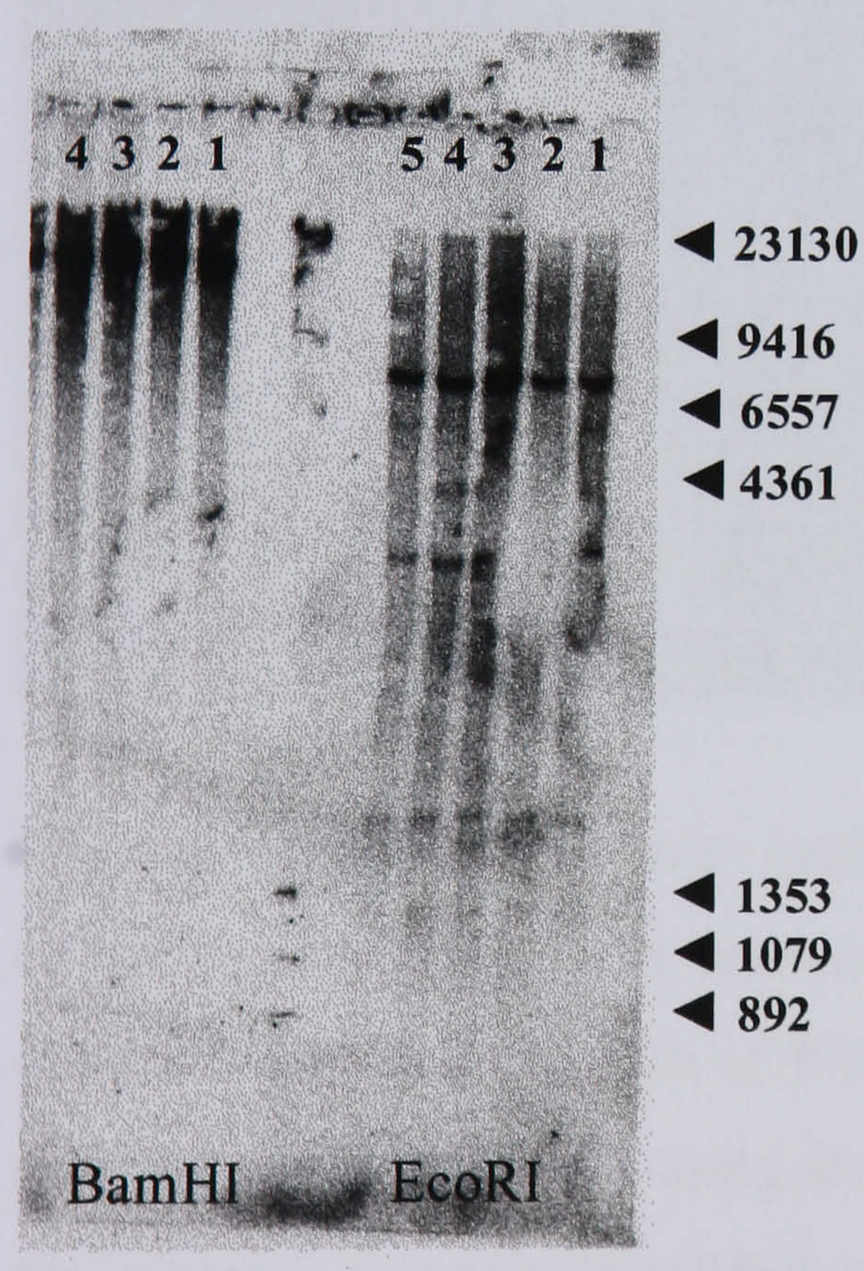


Fig 3.24e Southern blot of patient 22 and reference DNA hybridised with IGF2-R SB probe 5. Expected EcoRI fragment sizes were; 8175, 6515, 4035, 1730, 1559 and 1231 b.p. The expected BamH1digest fragment sizes were 17505, 16269, 14080 and 4110b.p. Restriction digest fragments of the expected sizes were seen in both patient and reference samples.

3.6.6 RACE PCR analysis of the *IGF2-R* (patient 22).

RACE PCR analysis of patient 22 was conducted to investigate the possibility that the observed translocation of 6q resulted in production of a fusion transcript consisting of exons of the *IGF2-R* and of a second unknown gene. On the basis of the FISH and Southern blot analysis an assumption was made that the translocation interrupted the *IGF2-R* between exons 1 and 10.

A sample of RNA from patient 22 was first run on an agarose gel confirming the presence of RNA but also revealing a relatively high level of contaminating DNA (**Fig 3.25**). 5' and 3' RACE ready cDNAs were then prepared using the patient RNA and also control placental RNA. First round PCR reactions were performed using the positive control TFR primers and 3' and 5' RACE ready placental cDNA. Bands of the expected sizes were obtained from both 3' and 5' RACE PCR reactions demonstrating the efficacy of the system (**Fig 3.26**).

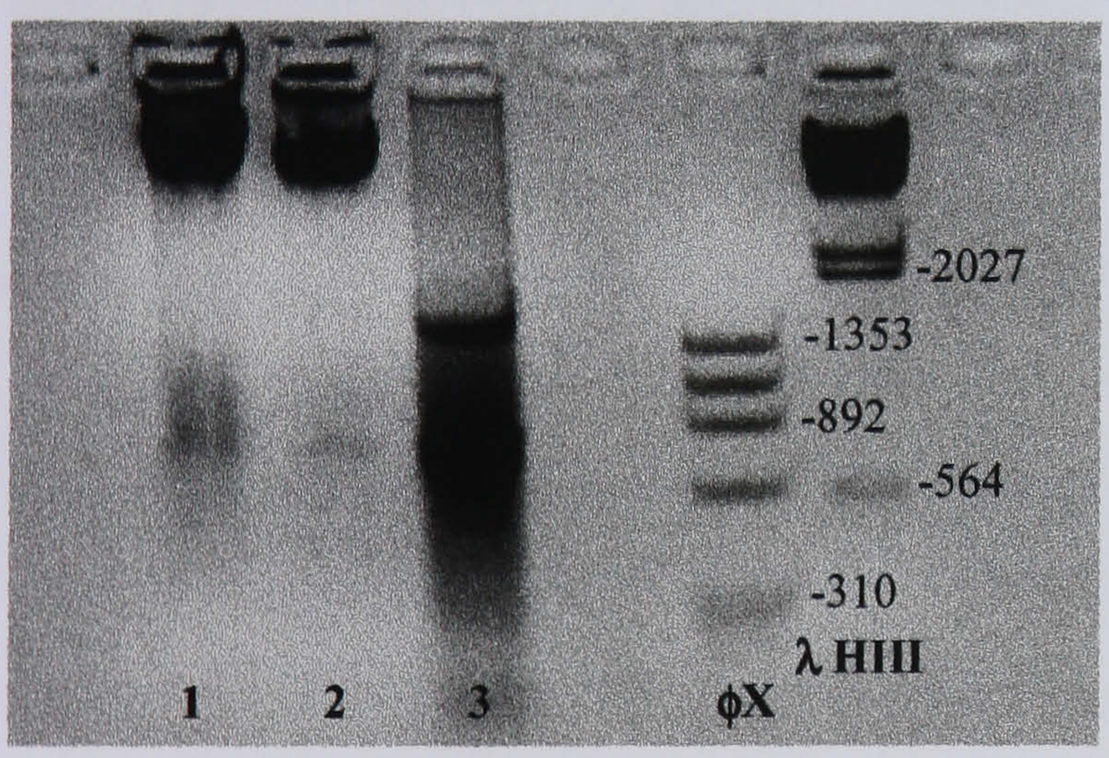


Fig 3.25 Assessment of patient RNA Sample by gel electrophoresis. (1) 3µl RNA from patient 22 (2) 3µl 1:10 dilution of RNA from patient 22 (3) 3µg reference placental RNA. The high molecular weight band seen in lanes 1 and 2 suggests that the patient Sample was heavily contaminated with DNA.

The same reactions were then performed using the patient RACE ready cDNA. No product of the expected size was seen for either the 5' or 3' reaction, probably reflecting the relatively low concentration of RNA in the patient's sample. To test the cDNA further PCR reactions were performed using forward and reverse *G6PD* and *β-Actin* primers. Products of the expected size were amplified with the *β-Actin* primers but not the *G6PD* primers, using 5' RACE ready cDNA prepared either from patient 22 bone marrow or placental RNA (**Fig 3.27**). This suggested that the SMART RACE cDNAs, while making effective templates for the amplification of sequences close to the 3' end of a gene, was less efficient for the

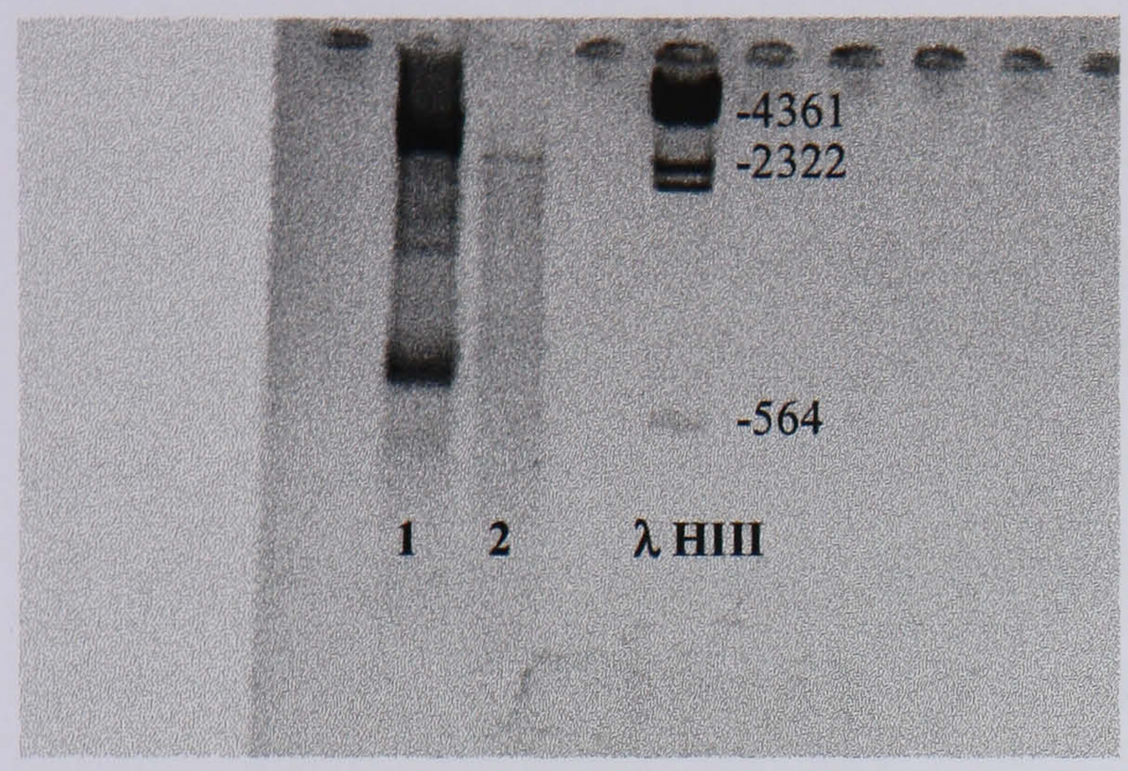


Fig 3.26 RACE PCR using positive control (*TFR*) primers to amplify cDNA made from a commercial placental RNA sample. (1) 3' RACE product showing the expected 2.9 Kb band and additional bands also present in the manufactures example image. (2) 5' RACE product showing the expected 2.6 Kb band.

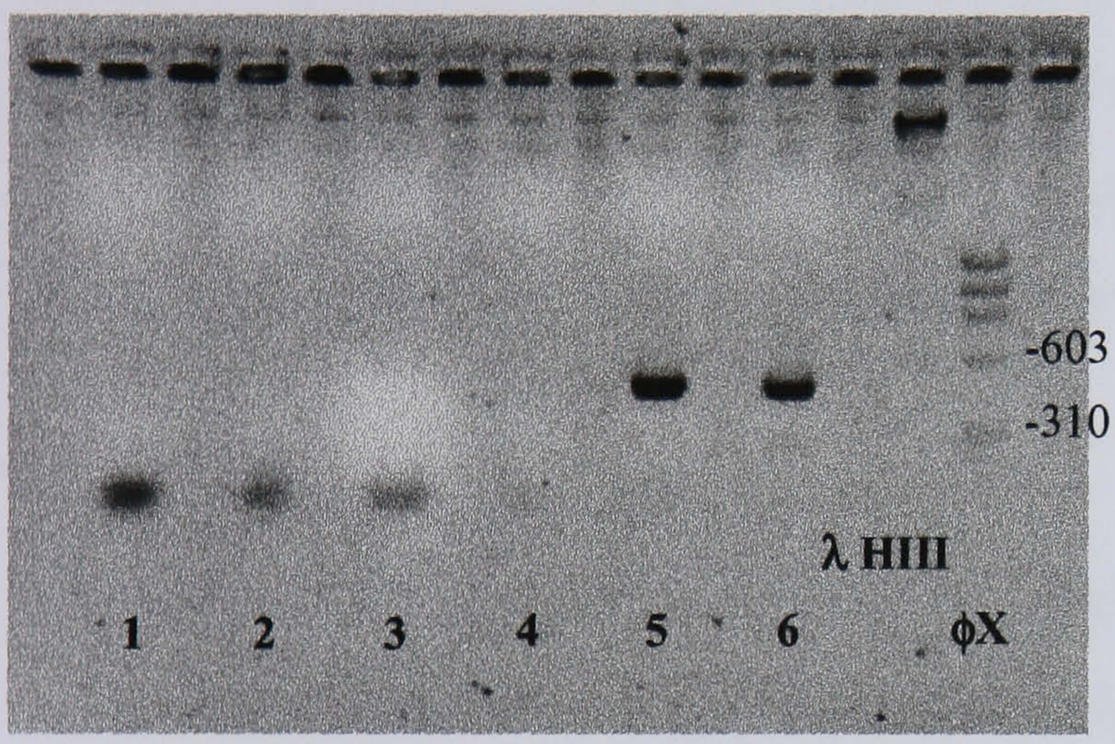


Fig 3.27 PCR using positive control primers to amplify patient RACE ready cDNA. Lanes 1-3 *G6PD* primers 4-6 *β Actin* primers. (1 and 4) H_2O, (2 and 5) 3' RACE ready cDNA, (3 and 6) 5' RACE ready cDNA. The expected product was amplified with the *β Actin* but not the *G6PD* primers.

detection of 5' sequences. Since both 5' and 3' cDNAs were primed from the 3' poly A tail (**see 2.14.1**) this limitation was expected, and furthermore the likelihood of detecting 5' sequences could be predicted to be inversely related to the distance between the 3' and 5' ends of the gene. Since the *IGF2-R* is a large gene (over 9Kb) and translocation was thought to have occurred close to the 5' end, cloning of the 5' part of any fusion gene would be expected to be inefficient. For this reason priority was given to 3' RACE reactions.

3' RACE was first attempted using the patient 3' RACE ready cDNA, the *IGF2-R* exon 1 primer and the universal primer mix for the first round PCR reaction. No visible PCR product was produced at this stage. A dilution of the first round PCR product was then used as a template for a second round of PCR with the nested universal primer and an exon 2 specific primer. The first round positive control 3' RACE product was also amplified using a nested TFR primer supplied with the SMART RACE kit.

Gel electrophoresis of the nested reaction from patient cDNA and *IGF2-R* gene specific primer resulted in a diffuse band (**Fig 3.28**). A heavy band of the expected size was obtained from the positive control (*TFR*) reaction. Both patient and positive control nested reaction products were then cloned using the TOPO TA cloning kit. Positive colonies were selected, grown, and digested with EcoR1 before checking for evidence of inserts (**Fig 3.29**). Inserts, of variable size, were seen in 12 of 14 positive clones obtained from nested 3' RACE of patient 22. All of 6 positive control colonies contained inserts of the expected size. Patient clones, containing inserts, were then sequenced. None of the 12 clones contained sequence of the exon 2 primers or any other part of the *IGF2-R* gene. Since the positive control reaction yielded cloned products of the expected size, indicating that there were no problems associated with the RACE PCR or cloning procedures, it was decided next to test the efficiency of the *IGF2-R* gene specific primers.

Combinations of *IGF2-R* exons 1, 2, 2b (internal to primer set 2) and 3 forward primers with exons 10, 8 and 7 reverse primers were then tested in PCR reactions using normal bone marrow cDNA that had already been assessed with *G6PD* primers. A positive PCR product was obtained when the exon 10, 8 and 7 specific primers were used with forward primers 2b and 3 but not 1 or 2 (**Fig 3.30**). This finding suggested that inefficient primer hybridisation might have caused the failure of the earlier RACE reactions to amplify *IGF2-R* sequences.

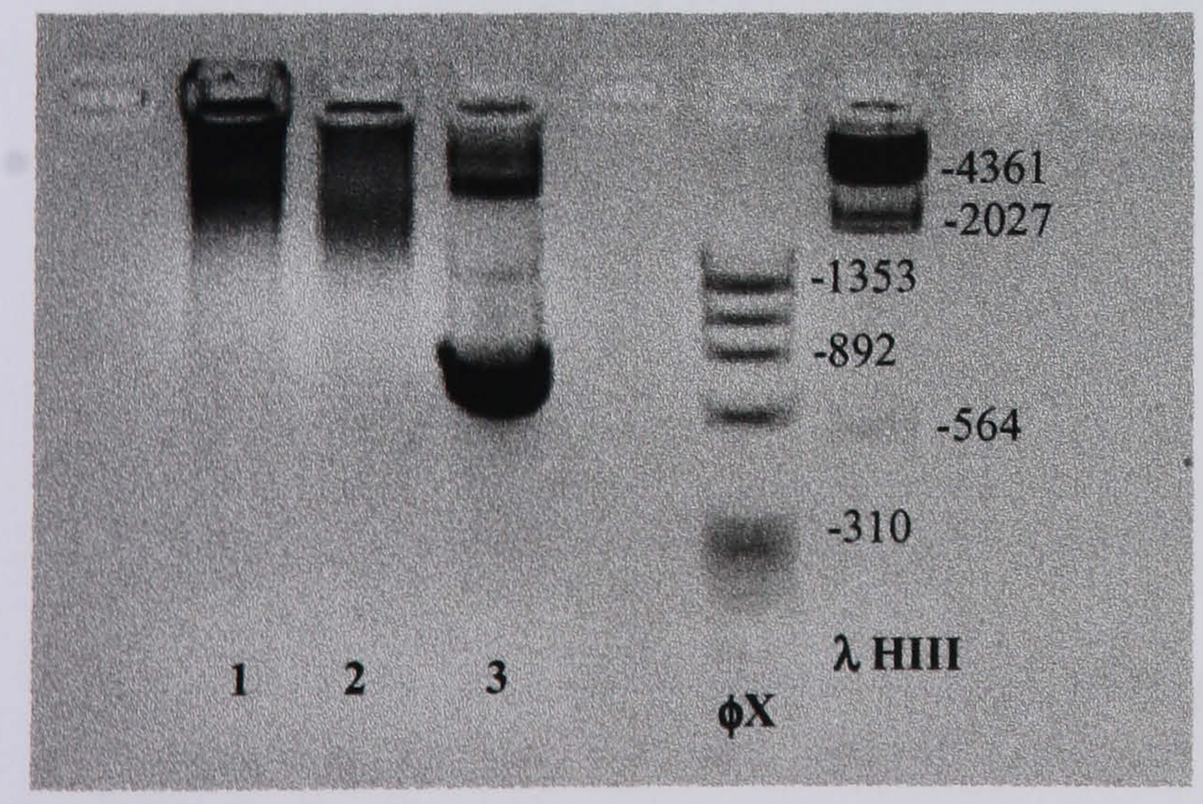


Fig 3.28 3' nested RACE PCR products. (1 and 2) from patient 22 cDNA using *IGF2R* gene specific primers. (3) using commercial placental cDNA and *TFR* gene specific primers.

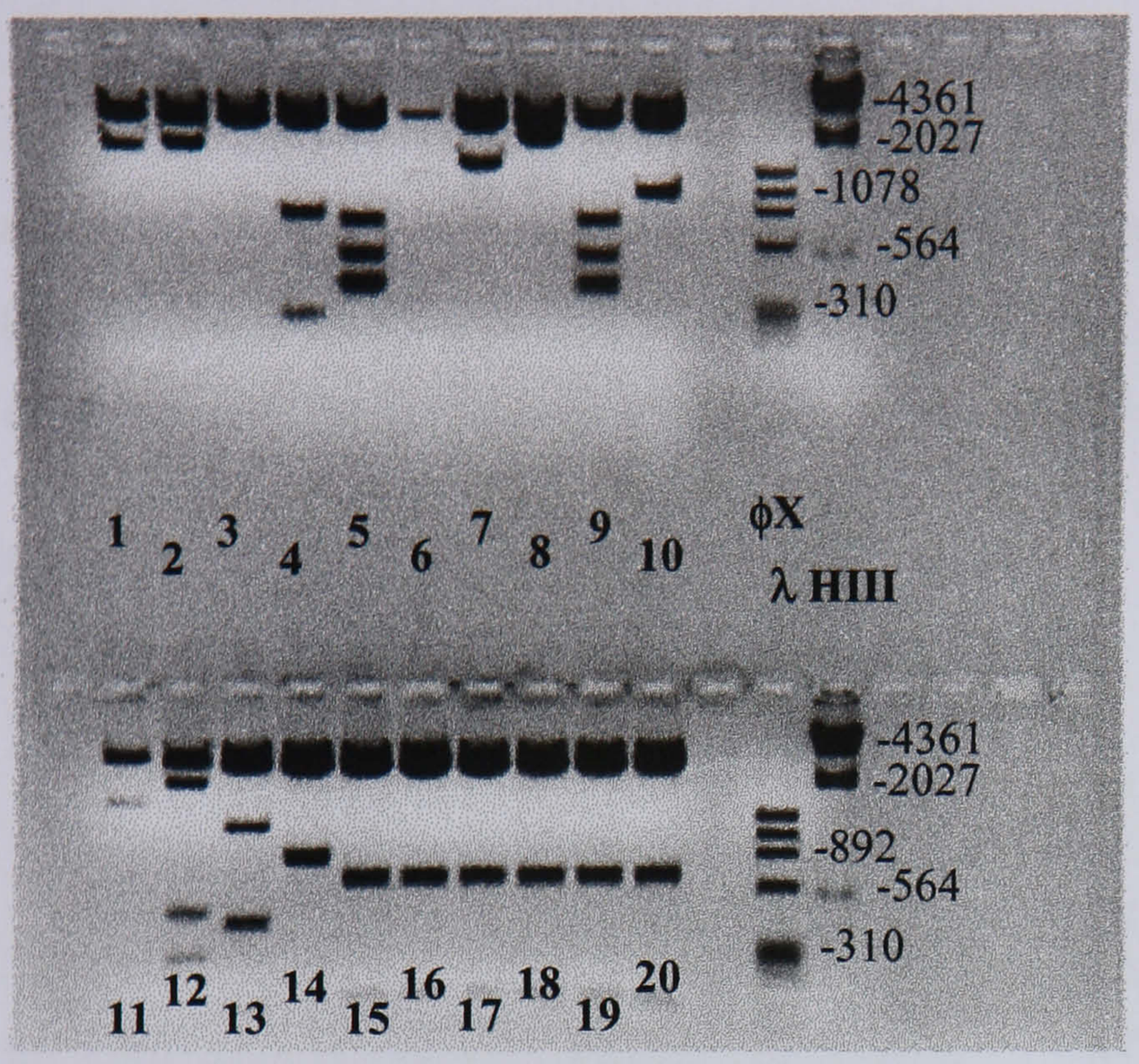


Fig 3.29 EcoRI digested clones of 3' nested RACE PCR products. Lanes 1-14 from patient cDNA amplified with *IGF2R* gene specific Primers. Lanes 15-20 from positive control (*TFR*) reaction.

The 3' RACE PCR reaction was then repeated using the patient 3' RACE ready cDNA as a primary template for amplification with the exon 2b primer for first round PCR and the exon 3 primer for nested PCR. No clear band resulted from the first round amplification but after the nested reaction, 3 major bands, between 600 and 1300 base pairs in size, were seen (**Fig**

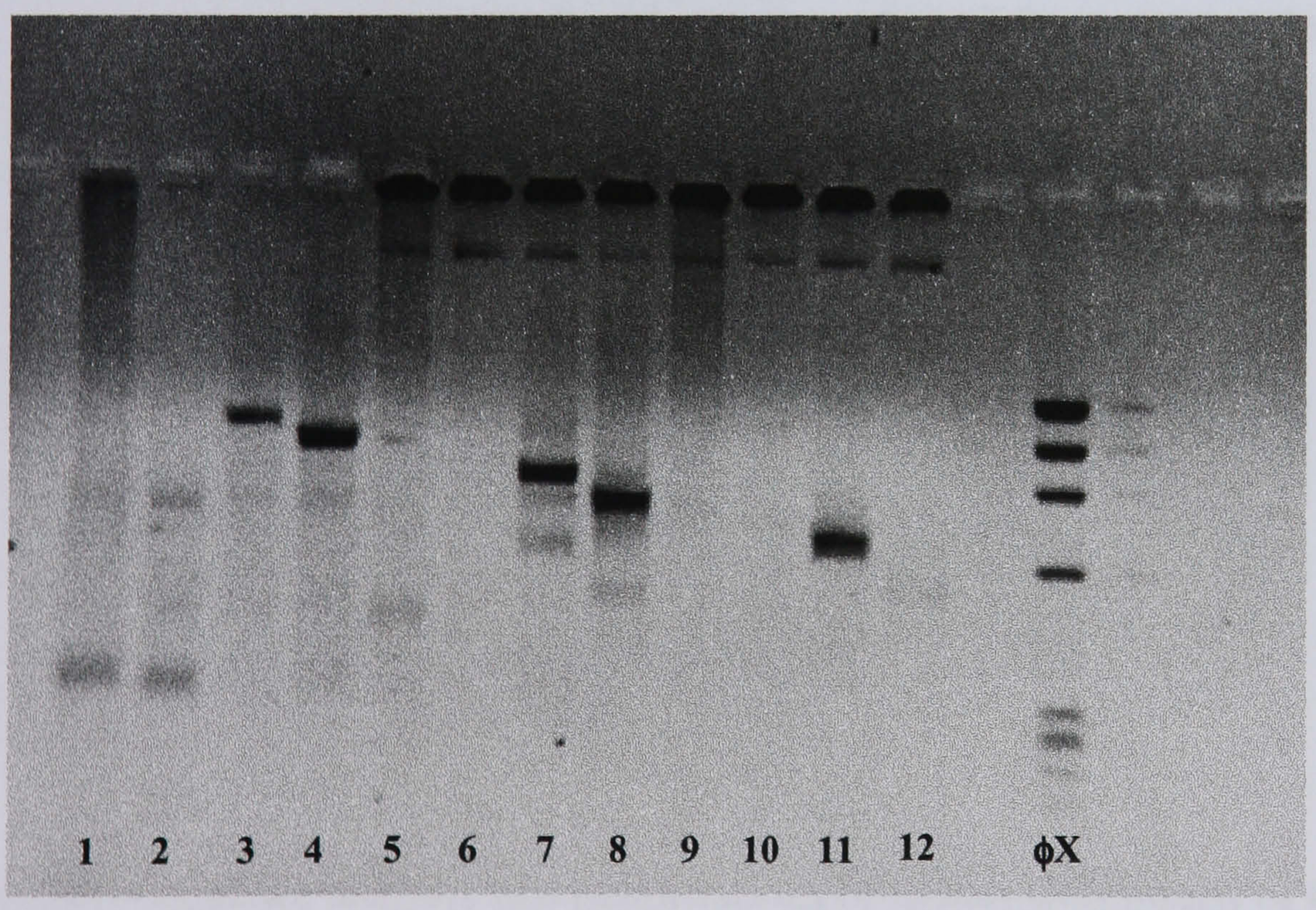


Fig 3.30 PCR of normal marrow DNA with paired *IGF2R* primers. Primer pairs were; (1) exon 1 and 10, (2) exon 2 and 10, (3) exon 2b and 10, (4) exon 3 and 10, (5) exon 1 and 8, (6) exon 2 and 8, (7) exon 2b and 8, (8) exon 3 and 8, (9) exon 1 and 7, (10) exon 2 and 7, (11) exon 2b and 7, (12) exon 3 and 7.

3.31). The nested PCR product was cloned as before but this time positive colonies were pre-screened in PCR reactions using the nested universal primer and the *IGF2-R* exon 3 primer (**Fig 3.32**). Colonies that gave a PCR product of over 500 bp in size were then grown, digested with EcoR1 and run on agarose gels to check for the presence of inserts (**Fig 3.33**).

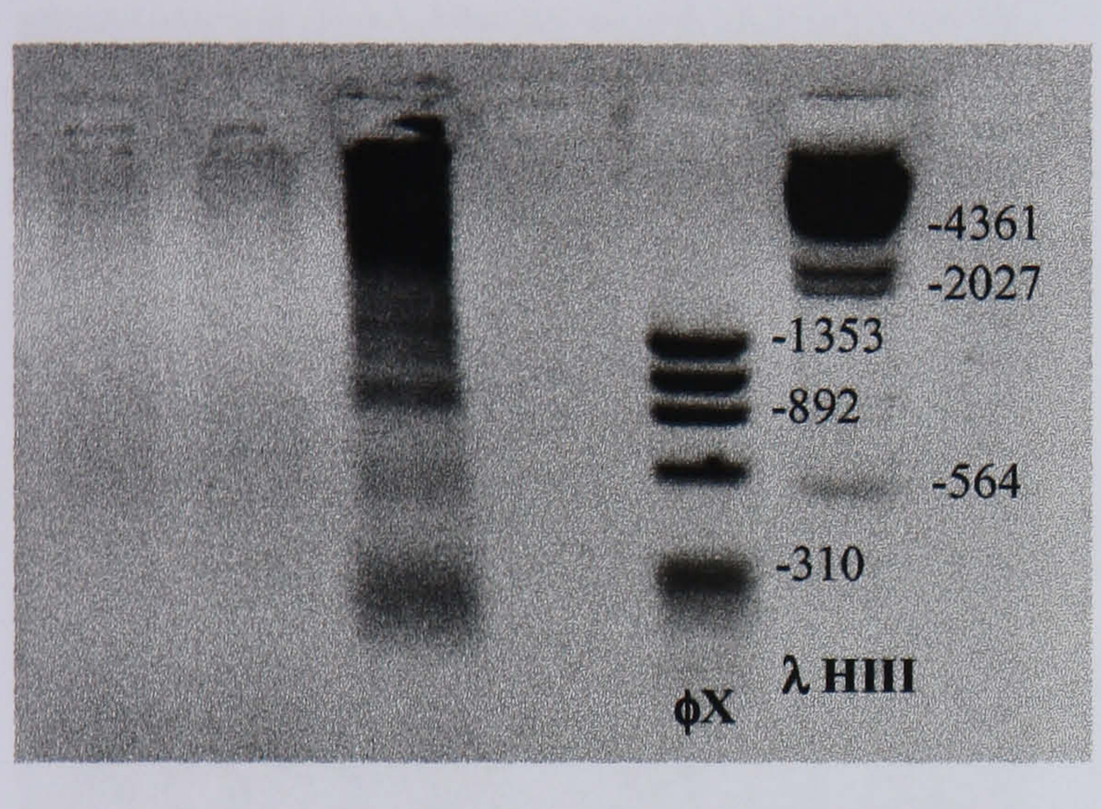


Fig 3.31 3' nested RACE PCR product amplified from patient cDNA with *IGF2R* exon 2b and 3 gene specific primers.

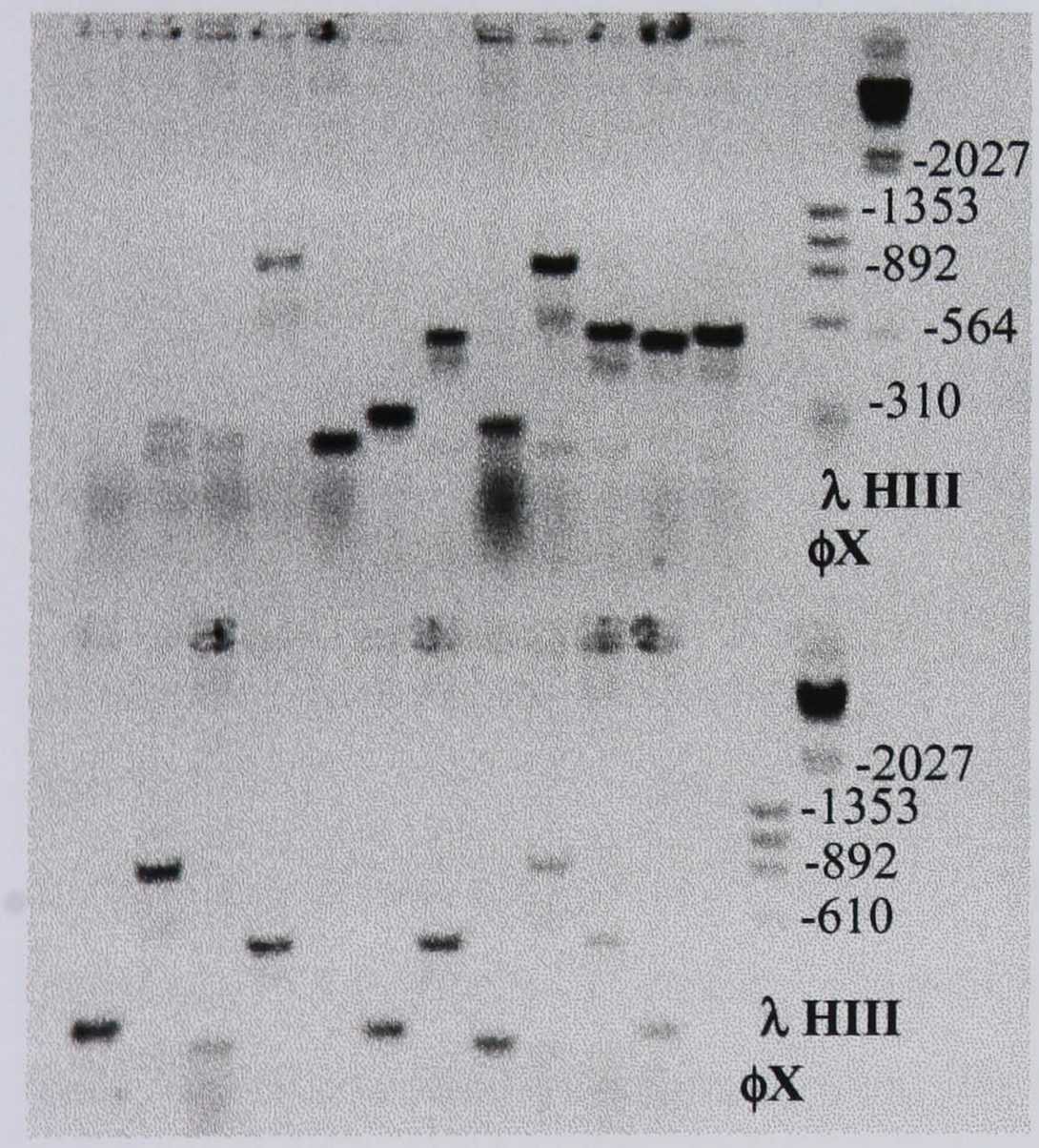


Fig 3.32 PCR of positive colonies cloned from the patient 3' nested RACE PCR product. Clones with inserts over 500 Kb in size were selected for further analysis.

Initially sequence analysis was performed on 20 clones with inserts of suitable size. Both the *IGF2-R* exon 3 specific sequence and NUP sequence joined to oligo dT sequences were found in pairs of forward and reverse reactions. Analysis of the intervening sequences revealed *IGF2-R* exon 3 and 4 sequence in all of the clones, in some genomic sequence

followed on from exon 4 or exon 5. Oligo dT primers were in some cases joined to intronic sequences downstream of *IGF2-R* exons; in others they extended into *IGF2-R* exon 12 or into unknown sequence that could not be identified on BLAST search.

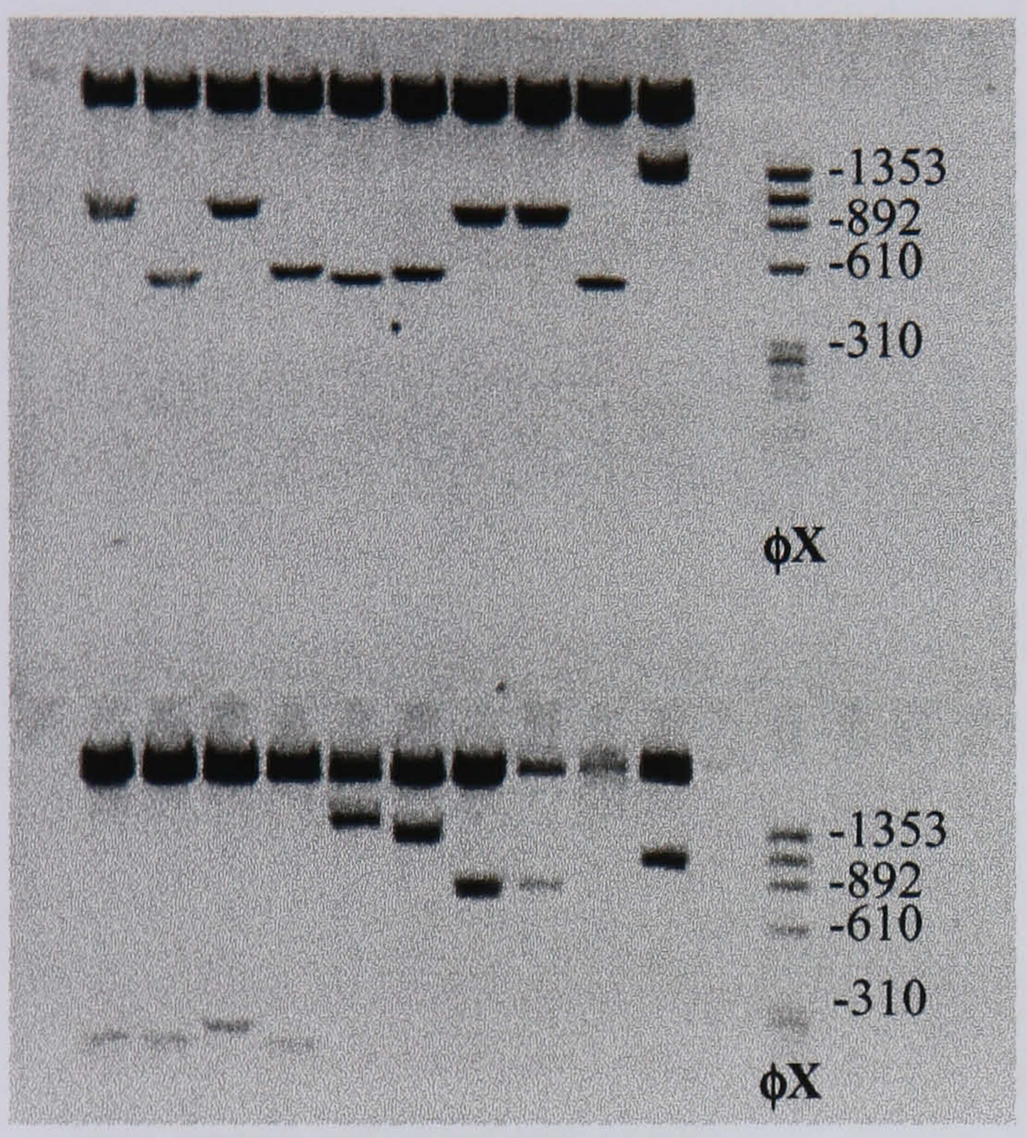
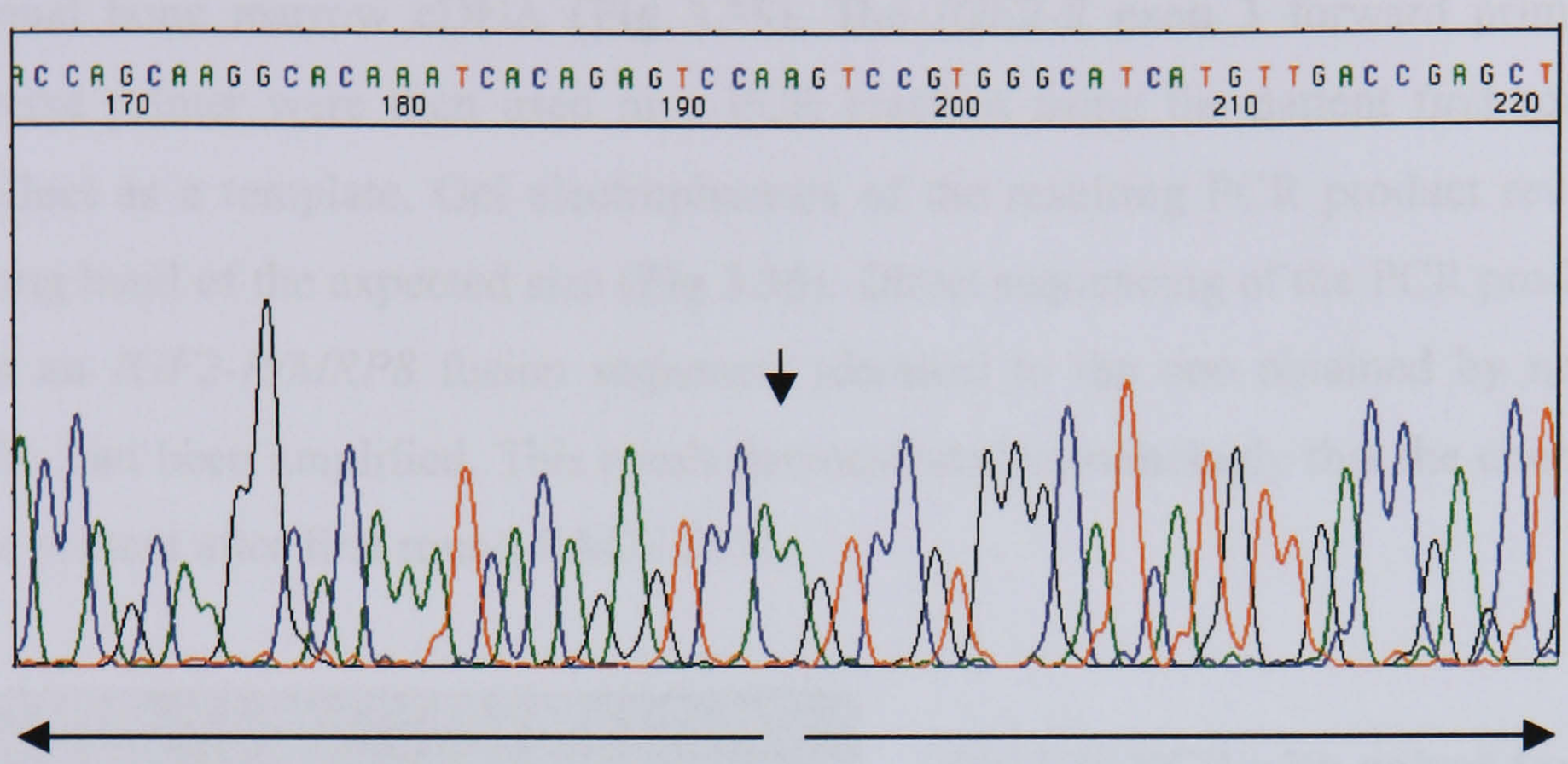


Fig 3.33 EcoRI digests of clones Containing 3' nested RACE products. Colonies were pre-screened by PCR to check insert sizes. Sequence analysis was performed on colonies with inserts of Over 500 Kb in size.

Further colonies were screened by PCR and after DNA preparation and analysis of EcoR1 digests by gel electrophoresis, 20 further clones were sequenced. As before the majority of clones consisted of *IGF2-R* exons 3 to 5 joined to either *IGF2-R* intronic sequence or unidentifiable sequences. However, two clones contained exons of a second gene, migration inhibitory factor-related protein 8 (*MRP8* or *S100A8*), fused to *IGF2-R* exon 3 sequence. The cloned sequences shared 100% identity with the published *MRP8* sequence from base pair position 39 (9 of exon 2) to the 3' end of the gene. The fusion with MRP8 occurred at base pair 530 (nucleotide 91 of exon 3) of the *IGF2-R* sequence and resulted in an out of frame sequence 3' the break point. A single base pair deletion had also occurred at position 12 of the *MRP8* exon 2 sequence. The sequence trace at the break point junction and for comparison the wild type *IGF2-R* exon 3and *MRP8* exon 2 sequences are presented in **Fig 3.34**.



gtgactctgt tttgagaagt gcaaccagat ctctcctgga attcaacaca
acagtgagct gtgaccagca aggcacaaat cacagagtcc agagcagcat
tgccttcctg tgtgggaaaa ccctg

IGF2-R
exon 3

Break point
b.p. 91-92

Break point
b.p. 8-9

Thymine residue at b.p. 12
deleted from fusion sequence.

gtggggc aagttccgtg ggcatcatgt tgaccgagct ggagaaagcc ttgaactcta
tcatcgacgt ctaccacaag tactccctga taaaggggaa tttccatgcc
gtctacaggg atgacctgaa gaaattgcta gagaccgagt gtcctcagta tatcagg

MRP8
exon 2

Fig 3.34 Sequence obtained from 3' nested RACE PCR of patient 22with *IGF2-R* gene specific primers. The 5' end of the *IGF2-R* (highlighted in red) was fused to 3' sequences of *MRP8* (highlighted in blue). Break points were between base pairs 91 and 92 of exon 3 of the *IGF2-R* and between base pares 8 and 9 of *MRP8* exon 2. Deletion of a Single nucleotide had occurred at position 12 in the *MRP8* exon 2 sequence (highlighted in green).

Forward and reverse and nested reverse *MRP8* specific primers from either side of the break point junction were then designed and shown to amplify product of the expected size from normal bone marrow cDNA (**Fig 3.35**). The *IGF2-R* exon 3 forward primer and *MRP8* reverse primer were then used in a PCR reaction using the patient first round 3' RACE product as a template. Gel electrophoresis of the resulting PCR product revealed a single strong band of the expected size (**Fig 3.36**). Direct sequencing of the PCR product confirmed that an *IGF2-R/MRP8* fusion sequence, identical to the one obtained by nested 3'RACE PCR, had been amplified. This result demonstrated convincingly that the chimeric sequence was present after first round RACE PCR.

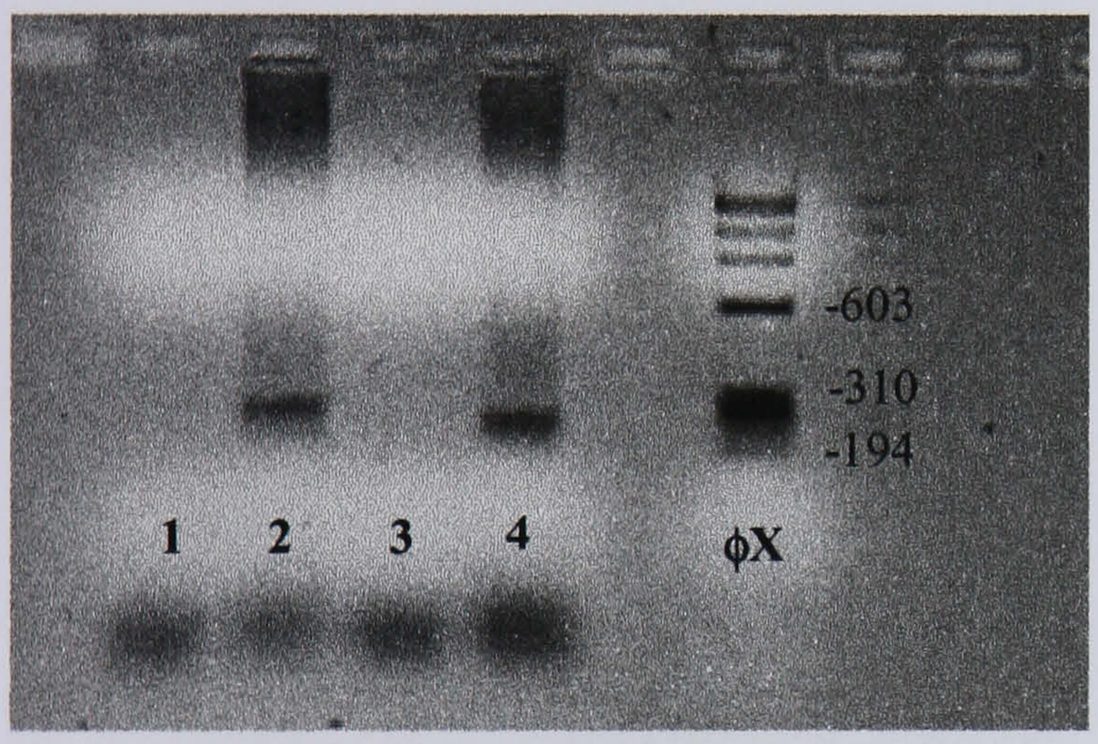


Fig 3.35 PCR with paired forward and reverse *MRP8* primers. (1 and 2) primers MRP8 forward and MRP8 reverse, (3 and 4) MRP8 forward and MRP8 nested reverse. (1 and 3) H_2O (2 and 4) cDNA from normal marrow.

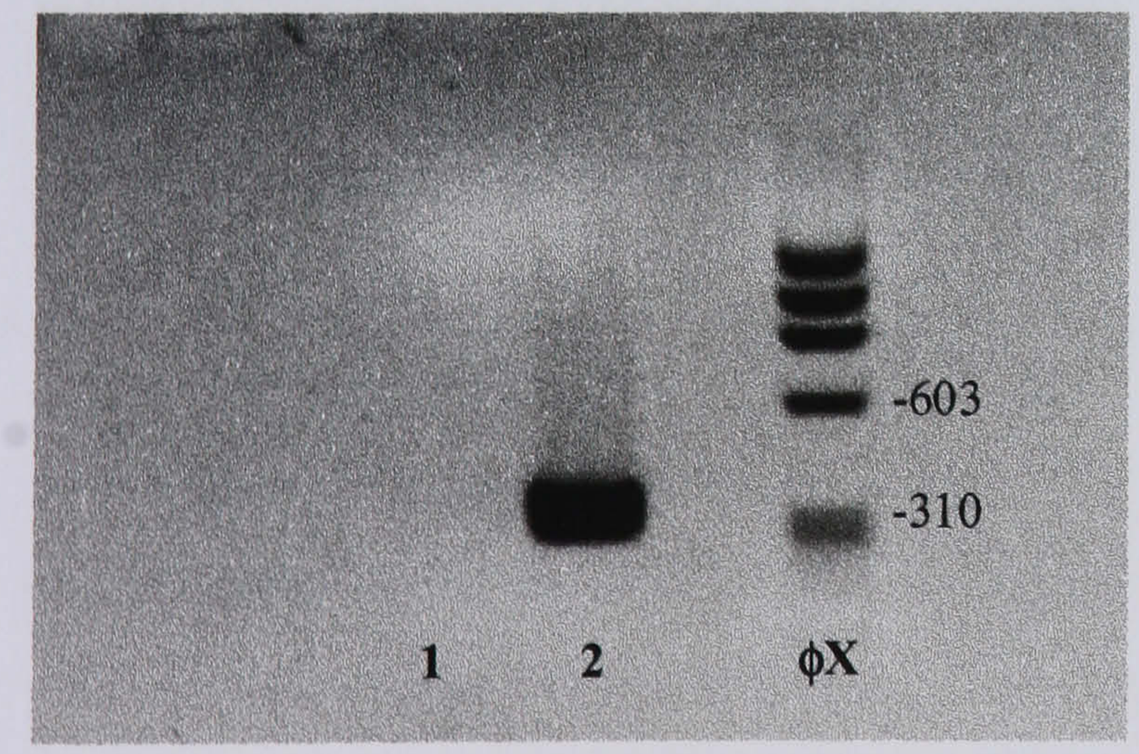


Fig 3.36 PCR with paired *IGF2R* exon 3 forward and *MRP8* reverse primers. template was; (1) H_2O (2) first round 3'RACE reaction product.

cDNA was then prepared from patient 22 RNA using random oligonucleotide primers. After confirming that a product of the expected size could be amplified using *G6PD* primers, the new cDNA was used as a template for paired *IGF2-R* exon 2b or 3 forward and *MRP8*

reverse primers in a PCR reaction. The same primer pairs were also used in an attempt to amplify the fusion sequence from the patient 3' and 5' RACE ready cDNA. No visible product resulted from any of the first round PCR reactions.

Dilutions of the first round product amplified with the *IGF2-R* exon 2b primer were then used as templates in nested PCR reactions using the *IGF2-R* exon 3 and *MRP8* nested reverse primer. As a positive control the same primers were used to amplify a dilution of the first round 3' RACE reaction product from which the fusion sequence was originally cloned. Gel electrophoresis of the nested reaction products resulted in a number of visible bands in each case, the positive control yielded a single strong band of the expected size (**Fig 3.37**). Bands that appeared identical in size to that seen in the positive control, were amplified from first round products made from the new cDNA and the 3' RACE ready cDNA.

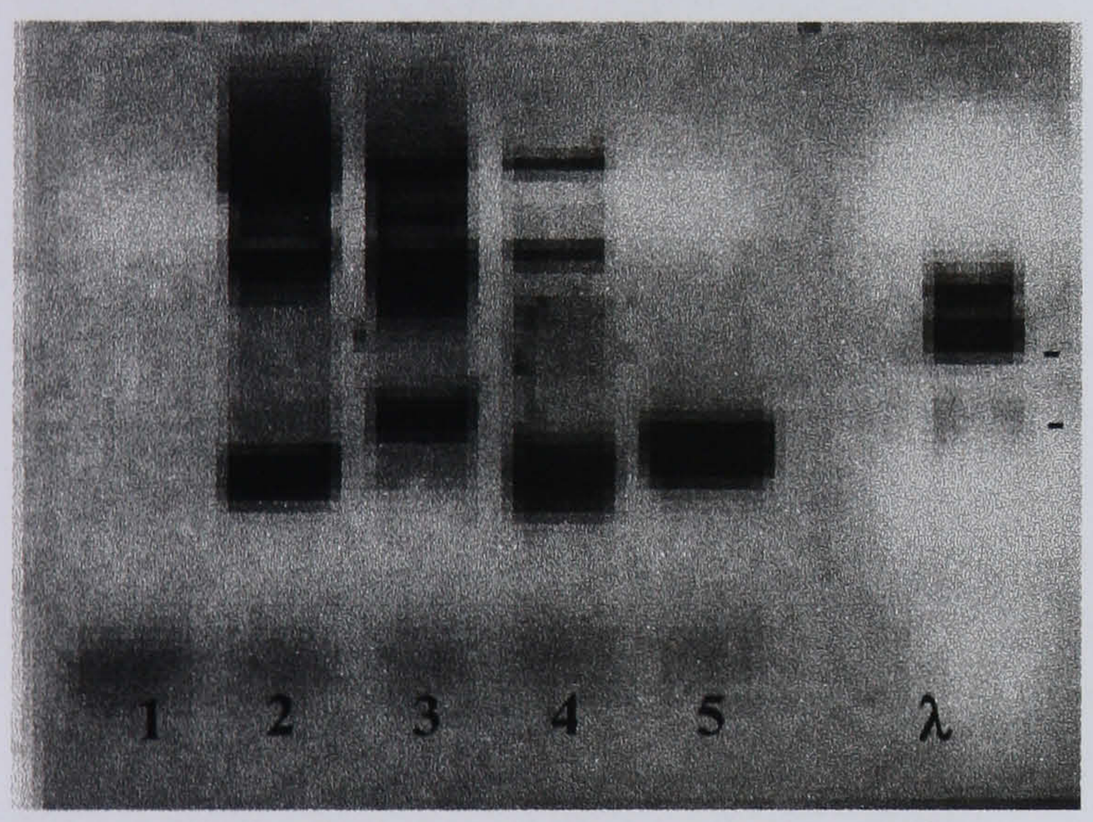


Fig 3.37 Nested PCR with paired *IGF2-R* exon 3 forward and *MRP8* nested reverse primers. Templates (1-4) were first round PCR products amplified with *IGF2-R* exon 2b and *MRP8* reverse primers from (1) H_2O, (2) 3' RACE ready cDNA, (3) 5' RACE ready cDNA, (4) cDNA prepared with random oligonucleotides. The template for 5 (positive control) was the first round 3' RACE reaction product.

In an attempt to produce a cleaner, more easily cloned PCR product, the nested PCR reaction was repeated using three different dilutions of the first round product amplified from the new cDNA, and shorter denaturation, annealing and extension steps. The positive control reaction was also repeated using the same program. Each nested reaction resulted in production of several bands including one apparently identical in size to the positive control band (**Fig 3.38**). Since bands additional to the one of expected size were less prominent in these reactions, one of the fresh products was cloned. Of 19 positive colonies amplified by PCR all contained products that were either the same size or slightly smaller than the positive control

band (**Fig 3.39**). Initially three bands of each size were sequenced directly but except for the primer sequences none contained sequences specific for *MRP8* or the *IGF2-R*. Additional clones were amplified by PCR and a total of 24 sequenced, of these none provided supporting evidence for the presence of an *IGF2-R/MRP8* fusion transcript.

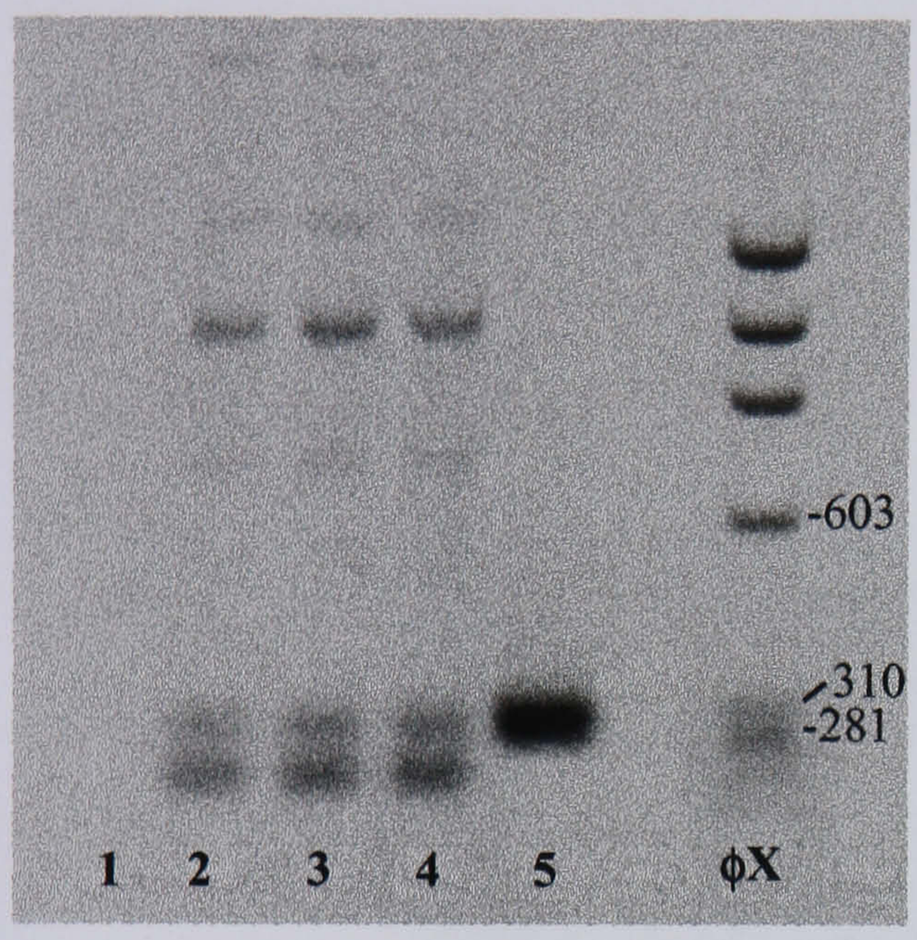


Fig 3.38 Nested PCR with paired *IGF2-R* and *MRP8* primers. Primers were *IGF2R* exon 3 forward and *MRP8* nested reverse. Templates were (1) H_2O, (2-4) dilutions of first round PCR product amplified with *IGF2R* and *MRP8* specific primers from Patient cDNA made with random primers, (5) first round 3' RACE PCR product.

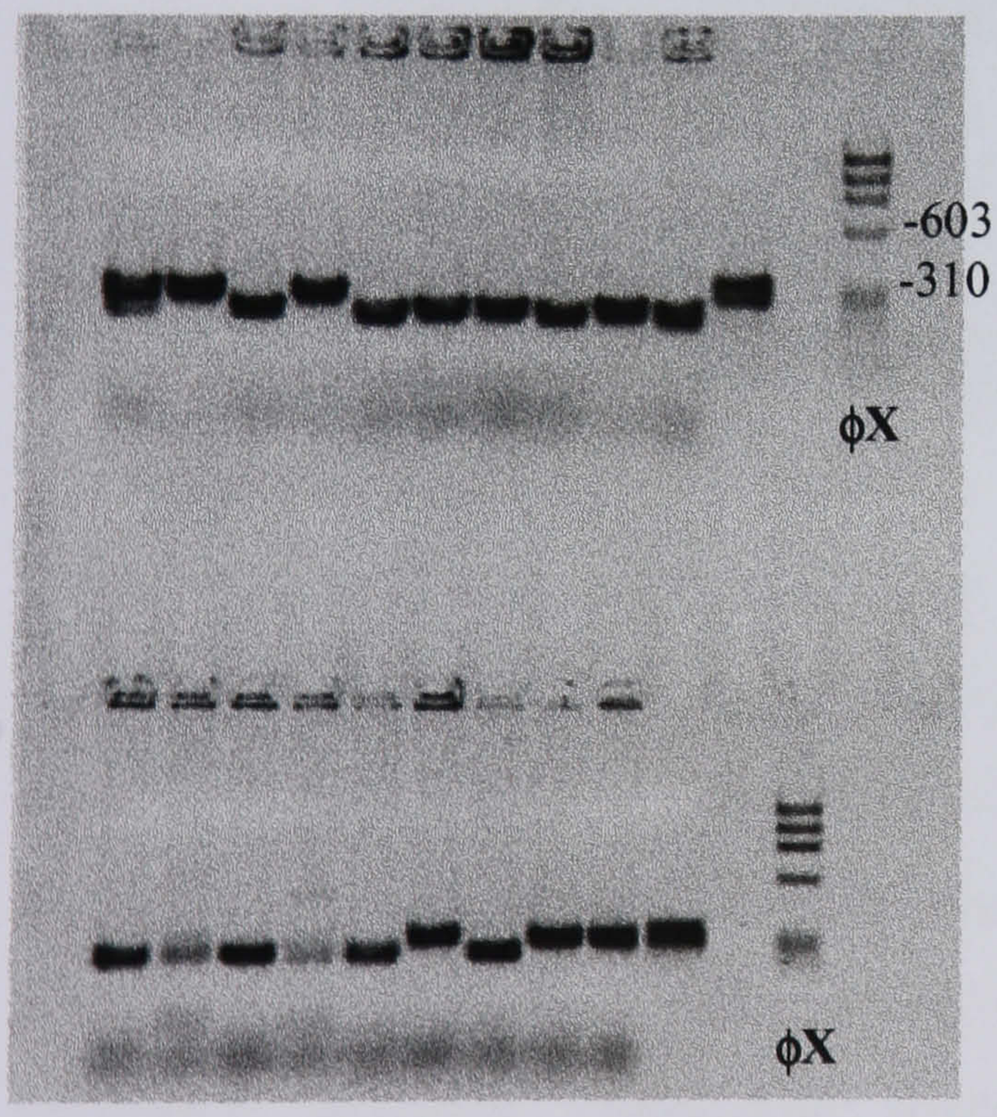


Fig 3.39 PCR amplification of positive colonies cloned from the product of nested RACE PCR with *IGF2R* and *MRP8* specific primers. All colonies Contained inserts of the expected sizes and were sequenced directly.

5' RACE PCR was also performed in an effort to find evidence for a fusion sequence. 5' RACE ready cDNA from patient 22 was amplified using the universal primer mix and either

IGF2-R exon 8 reverse or *MRP8* reverse primer oligonucleotides. While amplification with the *IGF2-R* exon 8 primer failed to produce any visible product, use of the *MRP8* reverse primer resulted in a single strong band (**Fig 3.40**). Direct sequencing of the *MRP8* 5' RACE product demonstrated that the band consisted of the *MRP8* wild type sequence including the 5' end of the gene. While failing to confirm that fusion between the *IGF2-R* and *MRP8* had taken place in patient 22 bone marrow, this result demonstrated that the 5' RACE ready cDNA was adequate for cloning the 5' end of a suitably small gene (418 bp). The contrasting failure to amplify *IGF2-R* sequences from the same cDNA confirms that the SMART RACE system was less effective for the amplification of the 5' end of large genes.

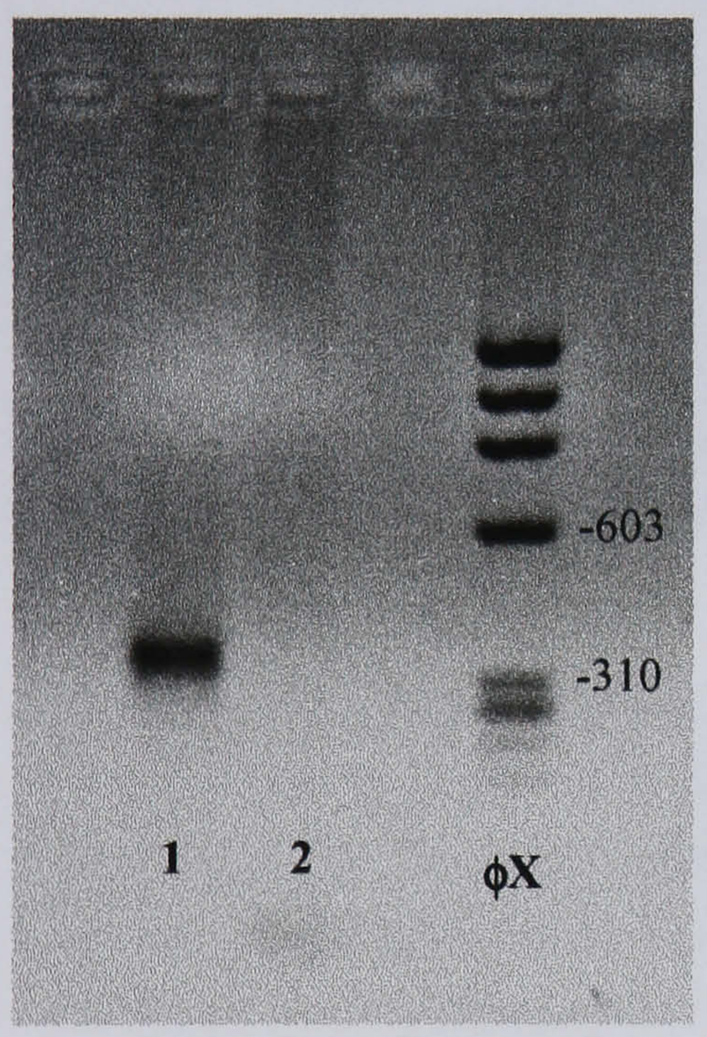


Fig 3.40 5' RACE PCR of patient 22.
(1) Using MRP8 reverse as the gene specific primer (2) Using IGF2-R exon 8 as the gene specific primer. The product In lane 1 was sequenced directly.

MRP8 was positioned on chromosome 1 band q21[282]. If translocation between the *IGF2-R* and *MRP8* had occurred in the patient 22 leukaemic clone, the maker chromosomes containing the telomeric region of 6q would also be expected to consist partly of chromosome 1. Chromosome 1 paint and RP1-57M24 were used to re-FISH metaphase cells from patient 22. No evidence for rearrangement of chromosome 1 was seen in any cells examined including metaphases where RP1-57M24 fluorescent signals were clearly positioned on the marker chromosomes rather than on normal copies of chromosome 6 (**Fig 3.41**). The fusion between *IGF2-R* and *MRP8* therefore either arose in the course of the first round 3' RACE reaction or resulted from a complex rearrangement involving insertion of a relatively small segment of chromosome 1 between part of 6q and a third chromosome.

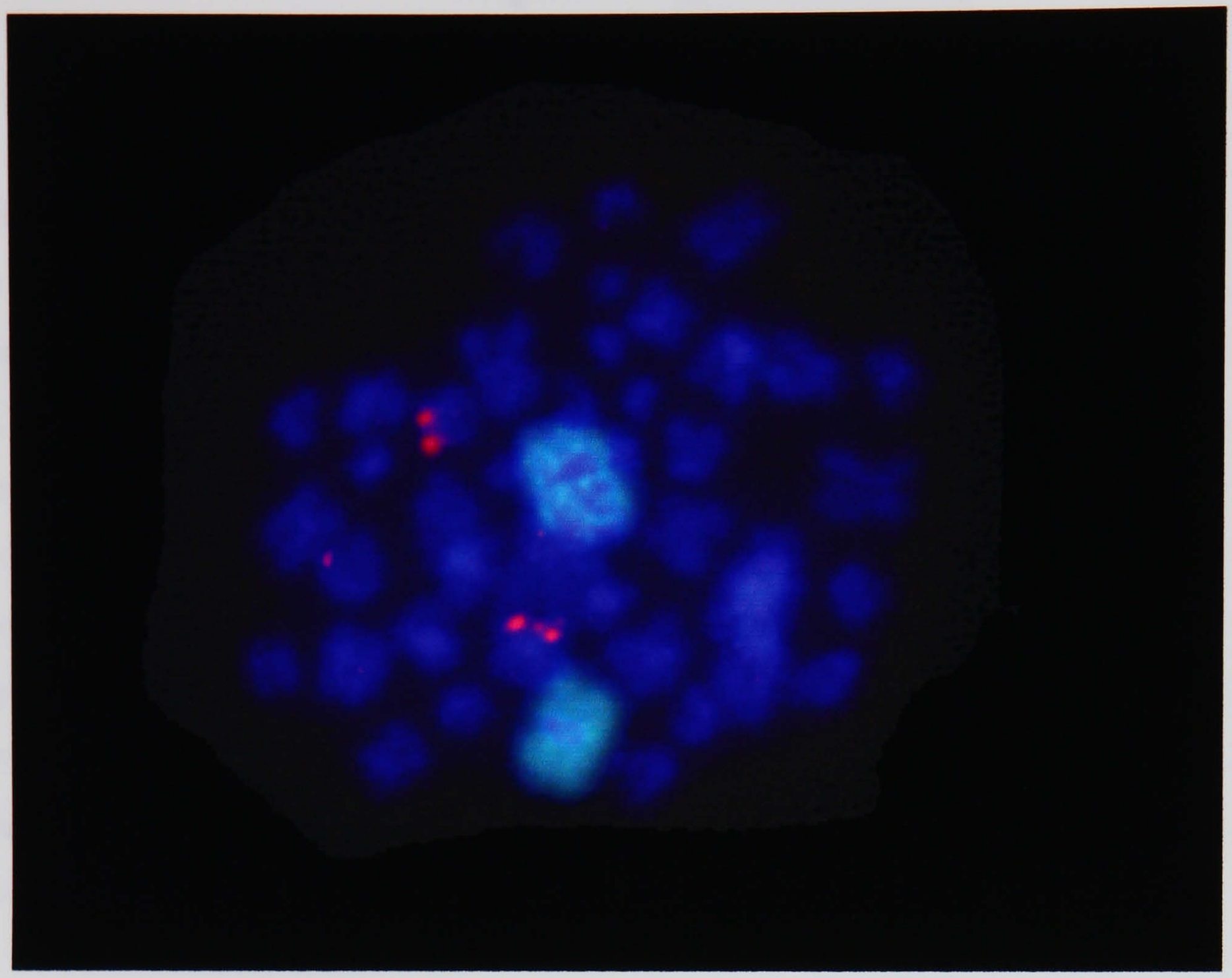


Fig 3.41 Example of a metaphase cell from patient 22 hybridised with a whole chromosome 1paint (green signal) and PAC RP1-167A14 (red signals). From chromosome morphology it is clear that RP1-167A14 is hybridising to the duplicated chromosome 6 translocation product, rather than to normal copies of chromosome 6, in this cell. Chromosome 1 appears to be unrearranged suggesting that it is not involved in the 6q translocation.

Chapter 4 (Discussion)

FISH analysis of acute leukaemia patients with cytogenetically detectable abnormalities of the long arm of chromosome 6 was performed with the principal aim of identifying one or more candidate tsgs. A secondary goal, the characterization of balanced rearrangements of 6q, developed unforeseen significance because of the high incidence of cryptic translocations. For the purpose of discussion, the translocations of 6q have been organised into three groups 1) cases of AML with breakpoints on partner chromosomes at the site of known oncogenes. 2) Cases of ALL or AML with breakpoints clustered within a 14 Mb region of q22-23 and 3) a single case of ALL with a complex karyotype involving both homologues of 6q and balanced and unbalanced translocations. Section **4.1** deals with the analysis of deleted cases, identification and investigation of a candidate tsg. Section **4.2** covers the three groups of balanced rearrangements.

4.1. Cases with deletion of 6q.

Twenty four of 34 leukaemia patients or cell cell lines analysed by FISH with PACs and BACs displayed loss of 6q. The pattern of deletions was open to different interpretations that depended on the number of tsgs positioned on the long arm of chromosome 6. The CDR identified in this study is based on a model in which a single leukaemia associated tsg is located on 6q, and this rests on two assumptions. Firstly, chromosomal rearrangements that involve translocation with a second chromosome as well as loss of DNA sequences are less likely to mark the position of tsgs than are simple interstitial deletions. In these cases formation of a fusion oncogene, deregulation of a gene near the translocation breakpoint or loss of tsgs on the partner chromosome may have driven clonal expansion. Secondly, it seems likely that at least some simple interstitial deletions confer no growth advantage but rather are neutral and carried by co-existent/unknown advantageous mutations. A low level of deletion occurs at all parts of the genome in haematological malignancies but only those regions showing consistent loss are thought to harbor tsgs contributing to leukaemia[251].

As outlined in **1.7** earlier analysis of patients with 6q deletions in this department led us to define two independent regions of deletion[256]. Of the two patients that defined the more telomeric region, one was included in this study (patient 22). Further analysis of this case revealed that both homologues of chromosome 6 were affected by either balanced or unbalanced translocation. Two other patients (23 and 24; Fig. **3.2**) showed loss of more telomeric regions of 6q but these cases also involved translocations. Of the cases investigated, none therefore, was shown to have a simple interstitial deletion confined to the more distal of the two previously identified regions. Disregarding patients 22, 23 and 24, (for the reasons given above), the remaining 21 cases were found to bear deletions that encompassed all or part of the more proximal of the two earlier defined regions. Twenty of the 21 deletions also overlapped with each other to give a 4.5 Mb CDR in band q16. Notwithstanding the possibility that the assumptions made in defining the new CDR were wrong, published LOH and FISH studies supported the existence of a leukaemia related tsg within this region of 6q (Fig **3.7**). Of seven published CDRs two fell entirely within our own region and three others overlapped with it.

4.1.1 Selection of a candidate tsgs from within the CDR.

The genomic region within the newly defined CDR contained 16 genes of which 7 coded for novel proteins of unknown function and 4 for genes that had not been characterized but were homologous to genes of known function. The 4 seemed unlikely to have a tumour suppressor role on the basis of the known function of the related genes. Of the remaining five genes *SIM1* and *POU3F2* (*BRN-2*) encoded transcription factors known to regulate development of neuroendocrinal tissues. The two transcription factors have not been reported to be expressed in haematological tissues though BRN-2 is also involved in melanocyte development. As discussed in **1.5** transcription factors regulating lineage development can also be tsgs. Consequently, the possibility that loss of *SIM1* or *BRN-2* contribute to leukaemia should not be dismissed until their expression in haematological tissues has been assessed. Of the two, *SIM1* seems the less likely candidate because hemizygous deletion was associated with early onset obesity in mice and a similar observation was made in a human bearing a translocation of *SIM1*. In neither case was malignancy reported[264]. A third gene, was recently identified

and shown to encode a receptor for melanin-concentrating hormone (*MCH2*). Like *SIM1* and *BRN-2*, *MCH2* was expressed predominantly in the brain, but did show significant expression in a minority of other tissues tested including lymphocytes[265,283]. In brain tissue, melanin-concentrating hormone is known to be involved in the control of appetite. The role of MCH2 in haematopoietic cells though is unknown, making its potential as a tsg difficult to evaluate at present.

Two other genes within the CDR, *GluR-6* and *CCNC* were functionally promising candidate tsgs (discussed further in **4.1.3**). Two facts argued against a role for *GluR-6* in leukaemogenesis; 1) *GluR-6* was not known to be expressed in haematopoietic cells and 2) mice homozygously inactivated for *GluR-6* were not reported to develop malignancies[284]. A tumour suppressor role for *CCNC* was also doubtful because analysis of cases of leukaemia with deletions of 6q failed to identify any inactivating mutations in the locus[285].

In the absence of a single candidate gene that clearly merited further investigation above others in the region, we compared published deletion data with our own CDR (as described in **3.4.3** and illustrated in **Figs 3.7 and 3.8**). As a result of this analysis a single gene, *GluR-6*, was unambiguously identified as the most promising of our candidate tsgs. Furthermore, a CDR derived from an LOH study conducted in our department by Dr Amani Sorour in cases of ALL (personal communication; data not shown), overlapped with the FISH CDR to give a consensus region containing only *GluR-6* as a candidate tsg.

4.1.2 Expression and mutation analysis of *GluR-6*.

As described in **3.5.1** the expression of *GluR-6* in haematopoietic as well as other tissues was investigated by RT-PCR. Using two different sets of primers one from the 5' and one from the 3' end of the gene, a product of the expected size was consistently amplified from normal bone marrow samples. Although GluR-6 transcripts were clearly more abundant in brain tissue, a visible product was amplified from marrow using a single round of PCR. Analysis of leukaemic samples and cell lines suggested that expression of *GluR-6* was more abundant

in lymphoid than myeloid cells. Preliminary analysis also suggested that *GluR-6* is widely expressed at variable levels in tissues outside the CNS.

Although any role for glutamate receptors in the haematopoietic system is currently speculative, the fact that *GluR-6* is transcribed in lymphocytes opens up the possibility that it might also protect against development of leukaemia. As discussed in **1.5** both heterozygous and homozygous inactivation of tsgs are thought to contribute to malignancy and a common scenario leading to homozygous loss of function involves point mutation of one allele accompanied by deletion of the second. To establish whether homozygous inactivation of *GluR-6* through this mechanism occurs in ALL, sequence analysis of the *GluR-6* exons and flanking intronic sequences was performed in patients and cell lines known to bear deletions of the region containing the *GluR-6* gene. Among 14 cases analysed a single base pair change resulting in an amino acid substitution was detected in exon 6. To date the possibility that this change might have been leukaemia-related, remains to be established. The base pair substitution was present in the patient's germ line DNA but was not seen in any of 20 additional normal samples analysed. These findings suggest that homozygous inactivation of *GluR-6* through a combination of deletion and point mutation is not a common occurrence in ALL patients carrying cytogenetic deletions of 6q. However, a base pair change present on one allele in the germ line, and retained in the leukaemic sample of an ALL patient with a 6q deletion, was not shown to be a polymorphism. The possibility therefore remains that point mutations of *GluR-6* result in altered protein function in rare cases of leukaemia.

No visible product could be amplified with primers specific for *GluR-6* exon 6 in one cell line (PEER). Exon 6 was amplified with ease in all other cases analysed and conversely, the other 16 exons of *GluR-6* could be amplified from the PEER DNA sample. One explanation for the repeated failure of PCR with primers for exon 6 in a single case is that microdeletion of the second allele resulted in loss of all or part of the target sequences. However, this possibility would need to be confirmed using another approach, such as hybridization of an exon 6 specific probe to a Southern blot of PEER genomic DNA. Alternatively, a polymorphism might have reduced the efficiency of primer annealing in this case. It is worth emphasizing that failure of PCR occurred only with one of the two cell lines analysed and not

with any of the 12 patient samples. Unlike the cell lines that are monoclonal, patient samples would all have included some normal haematopoietic cells in addition to the leukaemic clones. Complete failure of PCR, resulting from biallelic deletion, would therefore be unlikely to occur in the patient samples and homozygous inactivation through this mechanism would have remained undetected.

4.1.3 Evaluation of *GluR-6* and other candidate tsgs positioned on 6q, possible future studies.

Glutamate receptors can be broadly divided into metabotropic and ionotropic sub-types. The ionotropic receptors function as membrane bound ligand-gated ion channels that mediate the majority of excitatory neurotransmission in the brain[286]. The ionotropic receptors are further divided into three pharmacologically defined classes, named after their selective agonists, N-methyle-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate. The different ionotropic glutamate receptors are each associated with specific sub-units, and may be homomeric or heteromeric in composition. The sub-unit composition of receptors within each class can influence ligand affinity and the strength of ion flux induced by specific ligands. The known kainate specific receptor sub-units include GluR-5, GluR-6, GluR-7, KA1 and KA2.

In neurological tissues binding of glutamate receptors by their ligands results in a rise in intracellular Ca^{2+} levels but can also transduce signals through cytoplasmic proteins. Apart from neurotransmission the physiological consequences of glutamate signaling include the regulation of growth, migration and survival of developing neurons[287-289]. Stimulation of glutamate receptors has also been associated with the induction of apoptosis through Ca^{2+} mobilisation and through binding to the post-synaptic density protein 95 (PSD 95). PSD 95 links NMDA or kainate receptors to a variety of signaling cascades. In the case of GluR-6 specifically, binding of PSD 95 has been shown to lead to phosphorylation of MKK4 and MKK7, and to activation of JNKs and caspases followed by apoptosis[290]. PSD 95 is associated with synaptic junctions but has also been shown to function in T-cells[291]. The Drosophila homologue of PSD 95 (hDlg) is considered a tsg because inactivating mutations result in neoplastic overgrowth of some tissues[292].

Although it was assumed that glutamate signaling is restricted to the CNS, recently it has become evident that cells throughout the body possess glutamate receptors[293]. Tissues already shown to express glutamate receptors include osteoclasts and osteoblasts, megakaryocytes, keratinocytes, pancreatic islet cells, taste buds, lung, liver, heart, kidney and the enteric nervous system[294]. A number of studies have implicated glutamate signaling in haematopoietic cell function. *In vitro* glutamate was found to bind specific sites on T-lymphocyte plasma membranes and has also been shown to impair the activity of macrophages and lymphocytes[295-297]. In carcinoma patients elevated plasma glutamate levels were linked to a reduced lymphocyte response to mitogens and a high death rate[298,299]. Glutamate has also been shown to induce differentiation of HL60 cells and to be involved in the recall of the conditioned NK cell response, implicating glutamate signaling in a brain/immune system connection[300,301]. This, however, is the first study in which expression of a specific glutamate receptor has been demonstrated in haematopoietic tissue.

The known functions of GluR-6 include the induction of apoptosis through a p53 independent pathway and are therefore consistent with a tumour suppressor role. Although the function, if any, of this receptor in haematopoietic cells is unknown a protein involved in the transduction of pro-apoptotic signals (PSD-95) that lies immediately downstream of GluR-6 is present in lymphocytes. While the position of deletions in various studies, clearly suggest that loss of *GluR-6* contributes to leukaemia, mutation analysis produced only inconclusive evidence for homozygous inactivation. However, as discussed in **1.5** the level at which inactivating mutations can be detected in known tsgs in hematological tissues shows considerable variation. In some cases loss of heterozygosity alone seems to be sufficient to promote leukaemia while in others biallelic inactivation occurs through alternative routes. Deletions of the $p16^{INK4A}/p14^{ARF}$ locus for example are rarely accompanied by point mutations but more often by deletion or promoter methylation of the second allele. In this study homozygous inactivation through mechanisms other than point mutation of the coding region would not generally have been recognized, although failure of PCR for one exon in a cell line was suggestive for the presence of a second small intragenic deletion.

The *GluR-6*$^{-/-}$ mouse model provided no evidence for a tsg role for *GluR-6*. However, this cannot be considered conclusive because of differences in physiology between mice and men. Notably the relatively short life span of mice might mask any leukaemogenic effects of introduced mutations because there would be insufficient time to acquire the necessary additional genetic changes needed to develop recognizable malignancy. Production of double knock out mice, for example *p53*$^{-/-}$ *GluR-6*$^{-/-}$, would provide a more sensitive test of tumour suppressor activity. In this context, an interesting model would result from breeding *TEL/AML1* and *GluR-6*$^{-/-}$ animals because deletions of chromosome 6 are probably associated with t(12;21)(see **1.6**).

Against a background of only inconclusive support for an anti-leukaemic role for *GluR-6* it is worth considering both alternative assays to test its tsg potential and other candidate genes. First, attention should be paid to the genes from within the CDR defined by the FISH study. As outlined in **4.1.1,** 7 genes in the region encode proteins of unknown function that may later emerge as interesting candidates. The transcription factors *BRN-2* and *SIM1* should be assessed for evidence of expression in haematopoietic tissue before any further analysis is conducted. *MCH2* was shown to be expressed in lymphocytes and may be worth considering as a candidate for further analysis although its role in haematopoietic tissue has so far not been investigated.

CCNC has already been proposed as a candidate tsg because it was deleted in more than 90% of cases of childhood ALL with abnormalities of 6q, including a t(2;6)(p21;q15) without deletion visible at the cytogenetic level[285]. Unlike most members of the cyclin family that positively regulate the cell cycle through Rb phosphorylation, CCNC and its associated kinase (cdk8) form part of the RNA polymerase II holoenzyme complex. Recently CCNC/cdk8 was shown to regulate transcription by phosphorylation of the cdk7/cyclin H subunits of the transcription initiation factor IIH. Phosphorylation of cyclin H was found to have a dominant negative effect on cell growth[302]. Furthermore, expression of *CCNC* is upregulated by the nuclear hormone vitamin D, which is known to inhibit cell growth and induce apoptosis[303]. Functionally therefore *CCNC* is an attractive candidate tsg but SSCP analysis of 8 cases of childhood ALL with deletion of the region failed to identify mutations

of the second allele[285]. As it has already been argued in the case of *GluR-6*, loss of heterozygosity or homozygous inactivation of *CCNC* through a mechanism other than point mutation of the coding sequence, may contribute to leukaemia. Further investigations into the potential tumour suppressor role of *CCNC* would be justifiable on the basis of its position relative to leukaemia related deletions and its apparent role in the inhibition of cell growth.

As already discussed, the assumption that only a single leukaemia related tsg is positioned on 6q may be wrong. If two or more tsgs are present, the CDR might result from coincidental overlap of deletions that encompass more proximal and distal anti-leukaemogenic genes. On this basis, other genes in the 6q15-q21 region merit consideration as candidate tsgs. Positioned on the centromeric side of the CDR is *BACH2* a B-cell specific transcription factor contained within PAC RP1-253B9 that in this study was deleted in 17 of the 24 patients with 6q deletions. Treatment of lymphoid but not myeloid *BCR-ABL*- positive cell lines with the tyrosine kinase inhibitor STI571 resulted in a 2-10 fold upregulation of *BACH2* expression[304]. This result suggested that suppression of BACH2 function is associated with lymphoid transformation. Furthermore, enforced expression of *BACH2* in the Burkitt's cell line RAJI resulted in a reduction in both the number and size of colonies when plated on methylcellulose[305]. Southern blotting failed to reveal any rearrangements of *BACH2* in lymphoma or ALL patients[304]. However, the possibility that inactivating mutations or epigenetic silencing of *BACH2* occur in cases of leukaemia carrying cytogenetic deletions of the region remains untested.

Another master regulator of B-cell differentiation *BLIMP-1* is positioned telomeric to the CDR at band q21. BLIMP-1 is able to repress transcription through recruitment of HDACs and its ectopic expression was sufficient to induce differentiation of lymphoma cells to plasma cells[306,307]. Important targets of BLIMP-1 mediated repression include *c-myc* and *PAX5* both of which are upregulated in ALL as a result of translocation to antigen receptor loci (see **1.4**.)[308,309]. *BLIMP-1* is flanked by PACs RP1-132E6 and RP11-59I9, and was therefore deleted in between 15 and 18 of the patients analysed in this study. In view of its ability to suppress the expression of known ALL associated oncogenes, *BLIMP-1* would be a valid subject for further investigation in cases of lymphoid malignancy.

Several different approaches might be taken in order to establish the contributions that loss of candidate tsgs from the 6q15-q21 region make to haematological malignancy. Mutation analysis of cases bearing deletion of 6q might reveal evidence for homozygous inactivation as expected in the classical tsg model. Other techniques, such as Southern blot or methylation sensitive PCR, would be needed to establish the existence of biallelic deletion or epigenetic silencing at any of these loci. Quantitative RT-PCR and Western or Northern blotting could be used to investigate relative levels of expression of candidate genes in leukaemic cells and their normal counterparts. More global assays such as genomic array, differential display or proteomics could address the same question and would have the advantage that large numbers of genes could be investigated simultaneously.

The possibility that loss of heterozygosity of one or more genes in the region is leukaemogenic should also be taken into account. Evidence for such an effect might be obtained through functional assays. For example changes in the rate of growth or evidence for differentiation of ALL cell lines when transfected with a candidate gene would be suggestive for the rescue of loss of function. An assay of this type could also be used to demonstrate that point mutations truly result in loss of function. In particular, the relevance to leukaemia of the base pair change found in the germ line *GluR-6* sequence of one patient in this study could be investigated. More stringent evidence for effects of haploinsufficiency would depend on the demonstration in animal models that loss of function of a single allele is sufficient to increase the rate of malignancy.

4.2. FISH and molecular analysis of translocations.

Reported G-banded karyotypes that include balanced rearrangements of 6q are more common among myeloid than lymphoid malignancies[251]. Through our department we obtained 4 cases of AML with balanced translocations/inversions of 6q in which partner chromosomes were accurately identified. A fifth case was described as add(6q) and later shown to be a balanced t(3;6). Only two cases of ALL with translocations that were recognized on G-banding were obtained. However, a further 4 of the 25 cases of ALL

described as having del(6q) on G-banded analysis, were shown by FISH to involve balanced translocations. A further case had a translocation that also resulted in deletion of an interstitial part of 6q and 4 other cases appeared to be unbalanced translocations because the telomeric region (hybridizing to PAC RP1-167A14) was found to be deleted on FISH analysis (**Fig 3.2**). Two cases of myeloid leukaemia had unbalanced translocations one of which was noted in the G-banded karyotype (case 24). Unbalanced translocations will not be discussed further since they have been included in the analysis of deletions.

The high incidence of unrecognized translocations among ALL rather than AML patients is likely to reflect differences in the quality of G-banding typically achieved with the two types of leukaemia. The number of translocations of 6q revealed by FISH alone in cases of ALL, suggests that their incidence may have been previously underestimated. In this study there were no obvious differences in presentation features or outcome between ALL patients bearing translocations and deletions of 6q. However, numbers analysed were relatively small and a larger cohort of patients would be required to determine both the true incidence and any clinical association with the balanced rather than unbalanced rearrangements.

4.2.1 Cases of myeloid leukaemia with balanced translocations of 6q and break points at the site of known genes in partner chromosomes.

Only one of the five translocations of 6q found in cases of AML, the t(6;8) in patient 34, was found to be associated with a known fusion oncogene (*FOP-FGFR1*). In this case, FISH analysis suggested that *FOP* sequence fused to *FGFR1* was present on the derivative chromosome 6 only because no signal from RP1-167A14 was observed on the derivative 8 chromosome. As a consequence of the t(6;8)(q27;p11) originally cloned, both *FOP-FGFR1* and *FGFR1-FOP* were produced. The *FOP-FGFR1* fusion is thought to be oncogenic because its encoded protein retains the FGFR1 catalytic domain, however a leukaemogenic role for *FGFR1-FOP* has not been ruled out. As a result of translocation, *FOP-FGFR1* is located on the derivative chromosome 6[234]. The rearrangement analysed in this study therefore appeared to result in production of the *FOP-FGFR1* fusion only suggesting that, like *BCR-ABL,* it can be leukaemogenic in the absence of the reciprocal fusion product[115].

Remaining cases did not involve a recognised recurrent translocation therefore might result in production of novel chimeric oncogenes or in dysregulation of known or previously unknown oncogenes. With the exception of the t(2;6) and inv(6) found in patient number 32 the breakpoints on the partner chromosomes were within a band known to contain an AML associated proto-oncogene. If confirmed, the involvement of a known oncogene partner in any of these cases, might provide an opportunity to clone a new leukaemia related gene on 6q.

Of the three translocations that potentially involved known oncogenes, the t(6;11) was investigated and shown to interrupt the promiscuous oncogene partner *MLL*. The breakpoint on 6q clearly differed from those involving known *MLL* partners (*AF6* and *AF6q21*). Further molecular analysis was not, however performed in this case so the possibility that a complex rearrangement involving insertion of *AF6* or *AF6q21* (or a known MLL partner from another chromosome) into the 6q15 region has not been excluded. Identification of a rare new partner of MLL would have been of value because comparison between different partners may help elucidate the precise mode of action of the fusion genes[136].

A 6q abnormality, described as add(6q), was demonstrated by chromosome painting to be an apparently balanced translocation involving band 3q26, site of another promiscuous oncogene partner *EVI1/MDS1*. The *EVI1/MDS1* locus is unusual in that the two genes can be expressed separately or as a single transcript. *EVI1* encodes a transcriptional repressor that drives megakaryocyte differentiation[310,311]. Rare MDS, AML or CML(bc) associated translocations between EVI1/MDS1 and AML1, ETV6 or Ribophorin 1 result from t(3;21)(q26;q22), t(3;12)(q26;p13) and inv(3)(q21q26)/t(3;3)(q21;q26), respectively[61,62,312]. *EVI1* is also ectopically expressed in about 5% of cases of AML without cytogenetically detectable abnormalities of 6q26[313]. Since *EVI1* was known to be deregulated by different partner genes it seemed not unlikely that its expression was affected by the t(3;6)(q26;q25) identified in this study. However, a YAC containing *EVI1* (14e-e12), that had been shown to encompass the break point of 3 of 4 cases of inv(3)(q21q26)and failed to split on hybridisation to the t(3;6). This finding does not exclude the possibility that *EVI1* expression was affected by the t(3;6) because breakpoints at the locus are highly variable and may fall 5'

or 3' of the gene and outside the region encompassed by 14e-e12 [62,314,315]. Either expression studies, pulsed field gel electrophoresis or further FISH analysis would be required to eliminate *EVI1* as a candidate gene affected by this translocation.

A breakpoint within band 6q21 between PACs 429G5 and 66H14 was identified in the case of t(6;7)(q21;p15). This region of 6q contains the fork head transcription factor *AF6q21* which becomes fused to *MLL* in the t(6;11)(q21;q23). Band 7p15 is recurrently involved in the AML associated t(7;11)(p15;p15) resulting in the *NUP98-HOXA9 NUP98-HOXA11* or NUP98-HOXA13 fusion genes[97,316,317]. The novel t(6;7) may therefore be a rare variant of the *NUP98-HOXA* fusions involving *AF6q21* and a member of the *HOXA* cluster of genes. However, further FISH or molecular analysis would be needed to confirm this possibility. As with the other cases discussed in this section, characterization of the molecular lesion underlying a rare translocation will have little if any direct clinical impact, but may help clarify the mode of action of a known oncogene partner or pinpoint a new gene involved in normal haematopoiesis.

4.2.2 Cases of AML and ALL with break points clustered in the 6q22-q23 region.

In addition to the translocations discussed in **4.2.1** a further case of AML with a complex karyotype that included balanced rearrangements of both homologues of chromosome 6 was analysed. In this case neither the translocation partner break point (2q31) or inversion break points (6p13 and q22), were known to harbor leukaemia associated oncogenes. Though not identical, the translocation and inversion breakpoints on 6q occurred within the same region (bands q22-q23). Intriguingly, breakpoints in 5 of the 6 balanced rearrangements identified in cases of ALL, fell within the same region. A more detailed FISH map of the rearrangements of 6q22-q23 was constructed in the expectation that a new recurrent leukaemia related break point would be identified. Of the 7 breakpoints further analysis was not possible in two cases. Surprisingly of the 5 breakpoints mapped in detail, all occupied different sub-regions (see **Fig 3.19**).

The observation of clustered yet non-identical leukaemia related breakpoints in bands q22-q23 of chromosome 6 prompts two different explanations. 1) Several different rare new

oncogene partners, perhaps all belonging to a functionally related cluster of genes, might be positioned in the region. 2) The rearrangements might dysregulate expression of a single gene (or possibly multiple genes) through long-range effects on transcription.

The region of 6q containing the clustered breakpoints contained 91 recognised genes and covered just under 15 Mb, making it a relatively gene rich. Breakpoints for the 5 cases mapped in detail were narrowed to intervals of 1-2.5 Mb in size, in each case genes of known or predicted as well as of unknown function were present. Although it seems unlikely that five different genes involved in the pathogenesis of leukaemia would by chance be positioned in this relatively small region of 6q, interesting candidate oncogenes were found to be located within each breakpoint sub- region.

The translocation breakpoint from the AML patient no 32 (between PACs RP1-293L8 and RP1-179E13) contained a gene with homology to Protein Kinase-C inhibitor. Although in this instance not yet characterized, protein kinase-c inhibitors exert negative control over cell cycle entry and have anti-tumourogenic properties[269]. Also in the region were two genes that were homologues of known Drosophila developmental genes. The inversion breakpoint from the same patient (between RP1 199H14 and RP1-117D4) was positioned within a region containing three genes encoding cytokine receptors, *IL20RA*, *IL-22BP* and *IFNGR1*. As discussed in **1.1** dysregulation, inappropriate expression or modification of growth factor receptors is a characteristic of many malignancies. At least one of the three cytokine receptors, *IFNGR1* is expressed in haematopoietic tissues.

The breakpoint of the translocation found in patient 29 (between RP1-69D17 and RP1-131F15) was contained within a 2.5 Mb region encompassing several genes that could potentially be involved in malignancy. Notably *AKAP7* is essential for the function of PKA that in turn inhibits lymphocyte proliferation by down-regulating cyclin D3 and inducing $p27^{kip1}$ expression[271]. A second gene within the region, *CRISP 3,* is a target for the transcription factor Oct2 so probably it plays a role in terminal B-cell differentiation[272]. In this case the translocation was recognized on G-banding and thought to involve chromosome 19 band p13, the site of the *E2A* proto-oncogene. Demonstration of disruption to the *E2A*

sequence at the molecular level would present an opportunity to clone the break point and precisely determine which gene on 6q, if any, is involved in this translocation.

Detailed mapping of the translocation breakpoint in patient 27 defined a region (between RP1-55C23 and RP1-76H23) containing three genes involved in lymphocyte migration (*VNN1,VNN2* and *VNN3*). Interruption of genes in this cluster might interfere with lymphocyte infiltration of the thymus and hence perturb T-cell development[274,275]. Lastly, the breakpoint of the translocation found in patient 28 (between RP1-12L5 and RP1-42A6) fell within a region containing *c-MYB*, the only recognised oncogene in the 14.8 Mb region. The c-Myb protein is a sequence specific transactivating transcription factor expressed at high levels in immature hematopoietic cells [318]. c-Myb is known to play important roles in the growth and differentiation of haematopoietic cells and its loss of function was shown to increase the rate of apoptosis of T-cells in mice. Targets for c-Myb transactivation include *c-MYC*, *bcl-2* and a homeobox gene *GBX2*[319-321]. Activated forms of *c-MYB* were first isolated from chicken leukaemia viruses and subsequently truncating mutations of the wild type gene have been shown to stimulate malignant transformation in mice and to arise in human leukaemia [278,322,323]. Over-expression of *c-MYB* has also been reported in cases of leukaemia although its activation through translocation has not been reported[324,325].

In summary, the seemingly unlikely hypothesis that several different genes in the 6q22-q23 region can contribute to leukaemia, was strengthened on examining the gene content of the break point sub-regions. The region was gene rich and contained a concentration of genes specifically involved in haematopoietic development. However, involvement of the candidate genes in these patients will remain a matter of speculation unless further FISH and/or molecular analysis is performed.

As discussed in **4.2.1** the oncogene *EVI1* may be ectopically expressed as a result of translocations with breakpoints falling several hundred Kb outside the locus. Other genes such as *IL3* and *GSH2* may also be deregulated as result of relatively short-range position effects[99]. The mechanism by which oncogene deregulation occurs in these cases is uncertain but probably involves local disruption of higher order chromatin structure. The 5 breakpoints

defined in this study encompass a minimum distance of 5 Mb and a different mechanism leading to a long-range position effect would be required to bring about dysregulation of a single gene in the region.

That the same gene is dysregulated by all of the translocations is a theoretical possibility because long-range effects of translocations on expression have been demonstrated experimentally in *Drosophila*. A mutant of the brown eye colour locus (*brown*) was expressed at less than 2% of wild type levels through association with transcriptionally repressive heterochromatin. The repressive effect has been termed position effect variegation (PEV) and could be modified by translocations. Rearrangements that moved the mutant locus closer to the heterochromatin enhanced the trans-inactivation of *brown* while those that distanced it from heterochromatin counteracted the PEV[326].

Human genes including some involved in haematopoietic development are also trans-inactivated through association with centromeric heterochromatin. The related zinc finger DNA binding proteins Ikaros, Helios and Aiolos are thought to act as master regulators of B and T-cell development[327]. In the interphase nucleus Ikaros family proteins are concentrated at the centromeric heterochromatin clusters sometimes known as chromocenters. Using the approach of confocal microscopy and immunoFISH a striking correlation was reported between repression of genes with Ikaros binding sites (in this case TdT and λ5) and their association with the Ikaros-heterochromatin complex. Active Ikaros responsive genes were found elsewhere in the nucleoplasm providing an elegant demonstration of the role of chromatin movement in the control of transcription[328].

Modifications to heterochromatin mediated trans-inactivation of genes involved in human malignancy that result from chromosomal rearrangements, have not as yet been demonstrated. However, position effects of this type might account for the novel cluster of leukaemia-associated breakpoints observed in this study. Theoretically expression levels of any of the 91 genes in the region, or indeed of more proximal or distal genes, could be influenced by the translocations. An interesting candidate for such long-range position effects is *c-MYB* because its expression level varies during normal haematopoietic development and

the wild-type gene is sometimes over-expressed in leukaemia. Furthermore in ALL derived cell lines a correlation between high levels of *c-MYB* expression and the presence of 6q deletions has already been reported[324]. Two of the cell lines (RPMI-8402 and MOLT-4) have been analysed in this study and in both cases the telomeric boundaries of the 6q deletions were positioned 10-20 Mb proximal to the *c-MYB* locus. Any direct relationship between the deletions of 6q and high levels of c-MYB expression would therefore have to result from long-range position effects, although loss of trans-inactivation of the locus resulting from deletion of a more proximal gene is also a possibility.

Other potential targets of position effects include the genes discussed above that lie within the translocation breakpoint sub-regions and also genes positioned outside the sub-regions. Two genes (*Q9NYF8* and *MAP3K5*) that were not located within the sub-regions, encode genes involved in the regulation of apoptosis and therefore are potential candidates for dysregulation resulting from leukaemia related cytogenetic rearrangements. Though in principle, loss of transcription control mediated by chromocenters or other nuclear sub-domains might account for the cluster of translocation break points, evidence in support of the hypothesis would be difficult to obtain. It might be of interest to monitor expression levels of candidate genes, such as *c-MYB*, in future cases of leukaemia with rearrangements of 6q. The spatial relationship between candidate genes, on normal and rearranged chromosomes, and transcription factor associated nuclear sub-domains, such as the chromocenters, could also be investigated by confocal microscopy.

4.2.3 FISH and molecular analysis of a complex rearrangement of 6q (patient 22).

As described in **3.2.3** FISH analysis of patient 22 revealed complex rearrangements involving both homologues of chromosome 6. The most straightforward interpretation of the pattern of signals observed was that deletion of 6q, resulting from an unbalanced translocation, was accompanied by balanced translocation of the second homologue. As an additional complication one of the products of the balanced translocation appeared to have undergone duplication and some further rearrangements.

The breakpoint of the balanced translocation was mapped to a position between PACs 655C5 and 249F5. Using Blast Search the first four exons of the *IGF2-R,* covering a distance of more than 40 Kb were positioned on 249F5 while exon 5 and other more distal exons (spanning approximately 90 Kb) were found on 655C5. Since evidence for a split signal was not seen on hybridization with either 655C5 or 249F5 to patient 22 metaphases the translocation break point seems unlikely to have fallen outside the gene. Disruption of the *IGF2-R* could not be confirmed on Southern blot analysis, however this was thought to be for technical reasons. Based on the assumption that the translocation breakpoint fell within the first 10 exons of the gene, RACE PCR was performed.

With some difficulty a fusion transcript between the *IGF2-R* and a second gene (*MRP8)* was amplified from 3' RACE ready c-DNA prepared from patient 22 RNA. The amplified fusion transcript resulted in an out of frame sequence and therefore should not have produced a functional protein. Attempts to amplify the fusion sequence using paired *IGF2-R* and *MRP8* specific primers and an independently made cDNA from patient 22 were unsuccessful. FISH with a 6q probe lying telomeric to the breakpoint and a chromosome 1 paint also failed to provide supporting evidence for juxtaposition of the *IGF2-R* and *MRP8* sequences in this case. It is possible that a small segment of chromosome 1 including the *MRP8* locus became inserted next to the *IGF2R* as a result of a complex rearrangement involving not less than 3 chromosomes. It is also conceivable that the difficulty encountered in confirming existence of the *IGF2-R-MRP8* fusion arose because the chimeric transcript was unstable and quickly degraded. However on the basis of the data obtained the possibility that the fusion sequence was artificially produced in the course of the RACE reaction cannot be excluded. In conclusion the chromosomal rearrangements of 6q were likely to have resulted in biallelic inactivation of the *IGF2-R* and possibly in loss of function of one copy of a second gene, *MRP8*.

The IGF2-R is a multifunctional transmembrane protein that can bind the growth factors IGF1 and IGF2 in competition with the IGF1-R. Positive growth signals are transduced through the IGF1-R while the IGF2-R is thought to transport excess of these mitogens to the lysosomes for degradation[329]. Through a second binding site the IGF2-R interacts with the

mannose-6-phosphate moieties of certain glycoproteins including precursor TGFβ. Conversion of TGFβ from its inactive to its active form depends on IGF2-R mediated trafficking to the cell surface and in many tissues, including those of the haematopoietic system, active TGFβ transduces anti-growth signals through its receptors [330,331]. Recently the IGF2-R was shown to be the death receptor for granzyme B and therefore it may also play a role in immune surveillance. The IGF2-R has other important cellular functions but it is the above that have prompted expectations that it might act as a tsg.

In fact several lines of evidence suggest that the IGF2-R does play a tumour suppressor role in some tissues. Most convincingly 70% of hepatocellular carcinomas display LOH for the *IGF2R* locus and of these 25% were mutated for the second allele[332]. Missense mutations of the *IGF2-R* that alter ligand-binding affinity have been identified in various solid tumours.[40;41] In these cases mutations appeared to be monoallelic suggesting that haploinsufficiency of IGF2-R function, may be tumourigenic. Functional studies also support a tsg role for the *IGF2-R* because induced expression of the wild type gene was shown to suppress growth and promote apoptosis of a colorectal carcinoma cell line [333]. Transfection of antisense *IGF2R* cDNA into choreocarcinoma cells increased the rate of growth *in vitro* and also enhanced tumour growth in vivo[334].

This is the first report in which loss of function of the IGF2-R has been implicated in leukaemia. However, it is well known that the IGF system regulates proliferation and differentiation of hematopoietic cells (reviewed by Zumkeller 2002[227]). Hypersensitivity to IGF1 has been implicated as an underlying cause of PRV and loss of imprinting of the *IGF2* locus reported in cases of AML and MDS. *In vitro* IGF1 was shown to induce both antiapoptotic and proliferative effects in multiple myeloma cells[335]. Malignant lymphoblasts of childhood ALL were found to express components of the IGF system suggesting that they promote their own growth in an autocrine, paracrine or endochrine manner[336]. This last report is of particular relevance to the finding that the *IGF2R* is apparently homozygously inactivated in patient 22, who was a 10 year old presenting with ALL.

Cytogenetically detectable deletions of the region containing the *IGF2-R* (6q26) are not commonly reported in leukaemias. In this study only 6 cases (25%) would have included the *IGF2-R* compared with 20 (83%) for the most commonly deleted region. However, small deletions or point mutations, that would remain undetected on G-banding, might preferentially affect the *IGF2-R* locus. Deletions confined to the distal region of 6q have been reported to occur in lymphomas so it might be of interest to investigate the involvement of this tsg in a range of lymphoid malignancies[337]. Because of the large size of the *IGF2-R*, mutation analysis of the entire coding sequence might be viewed as impractical. In solid tumours mutations were found within the extracellular domain that includes the IGF and M6P binding sites; so analysis of exons encoding this part of the gene could be considered for an initial investigation[338,339].

Though unconfirmed the RACE PCR results suggest the possibility that not only the *IGF2-R* but also *MRP8* was inactivated as a consequence of chromosomal rearrangements in patient 22. MRP8 is one of a cluster of genes positioned on chromosome 1 band q21 that code for related calcium binding proteins[282]. The calcium binding proteins appear to be involved in processes such as cell cycle progression and differentiation. *MRP8* in particular is expressed in macrophages associated with chronic tissue inflammation[340]. Though function of MRP8 is not inconsistent with a role in leukaemia further investigation of this gene would not be justified on the basis of the data obtained in this study alone.

Conclusions.

FISH analysis of cases of ALL with cytogenetically detectable rearrangements of 6q lead to the identification of a new CDR and candidate tsg (*GluR-6*). *GluR-6* encodes one of a number of ionotropic glutamate receptor sub-units that was demonstrated for the first time to be expressed in haematopoietic tissue. One of 14 patients with leukaemia related deletions that included *GluR-6* was found to carry a base pair substitution in the non-deleted allele. Although the base pair substitution resulted in a change in amino acid sequence, it was also present in the patient's remission sample so its relationship, if any to the patients leukaemia, is unclear. Further investigations of the involvement of *GluR-6* as well as a number of other genes in the region (*CCNC, BAC-2* and *BLIMP-1*) in leukaemia would be justifiable.

Eleven leukaemia related translocations affecting 6q were also analysed. None of the translocation breakpoints fell within the CDR, four involved bands on partner chromosomes known to contain proto-oncogenes and seven clustered within approximately 15 megabases of 6q22-q23. On detailed analysis the clustered breakpoints were found to be non-identical suggesting that either multiple-oncogenes are positioned within this part of 6q or that these rearrangements contribute to leukaemia through a novel mechanism. Complex rearrangements of 6q in a single case of childhood ALL were consistent with biallelic loss of function of the IGF2-R. Though inactivation of the IGF2-R has been associated with several solid tumours this is the first evidence to suggest that it may also operate as a tsg in ALL.

Appendix 1

Materials (not included in chapter 2).

Chemicals were obtained from BDH unless otherwise stated.

AGB Buffer (50X).

242g trisma base

57.1 ml glacial acetic acid

100ml 0.5M EDTA pH 8.0

made up to 1L with DDW

AHC Medium.

1.7g bacto-yeast extract

10g caseine hydrolysate

5g $(NH_4)_2SO_4$

10ml (2 mg/ml) adenine sulphate

to 900 ml with DDW and autoclave

before use add 10% volume of filter sterilised 20% glucose and antibiotics

AHC Agar.

As for AHC medium but glucose and 15g bacto-agar added before autoclaving

Solid media is liquidised by microwave and cooled to 50^0C before addition of antibiotics

Church Hybridisation Buffer.

500ml 1 M Na_2HPO_4 pH 7.2

10g Bovine Serum Albumin

70g Sodium Dodecyl Sulphate

2ml 0.5 M EDTA pH 8.0

Denaturing solution.

1.5 M NaCl

0.5 M NaOH

Depurination solution.

20 ml of HCl in 1 litre DDW

DNA loading buffer.

50% glycerol

0.2% bromophenol blue

0.2% xylene cyanol

0.1 M EDTA pH 8.0

DnD.

1 M DTT

90% DMSO

10 mM Potassium Acetate

GDIS solution.

20 ml 10% triton-X-100

10 ml 10% SDS

3.33 ml 3M NaCl

1M 1 ml Tris pH 8.0

200 µl 0.5M EDTA

to 100 ml with DDW and filter sterilize.

GTE buffer.

50 mM glucose

25 mM Tris pH 8.0

1 mM EDTA pH 8.0

Luria-Bertani broth (LB medium).

10g bactotryptone

5g bacto-yeast extract

10g NaCl

to 1L with DDW

LB agar.

1.5% bacto-agar in LB

sterilized by autoclaving

solid media liquidised by microwave and cooled to 50^0C prior to addition of antibiotics

Neutralising solution.

1.5 M NaCl

0.5 M Trisma base pH 7.2

PEG (20%).

40g PEG

29.22g NaCl

dissolved in 100 ml DDW, filtered and made up to 200 ml final volume.

Phenol/chloroform/isoamyl alcohol (PCIA).

25 ml phenol pH 8.0

24 ml chloroform

1 ml isoamyl alcohol

SOB.

20g bacto-tryptone

5g bacto-yeast extract

0.5g NaCl

10 ml 250 mM KCL

adjusted to pH 7.0 and made up to 1L with DDW

SSC (20X).

3M NaCl

0.3M sodium citrate

adjust to pH 7.0 with NaOH

TBE (5X).

27g Trisma base

13.75g boric acid

10 ml 0.5 M EDTA pH 8.3

to 500 ml with DDW

TE buffer.

10 mM Tris pH 8.0

1 mM EDTA pH 8.0

TFB.

0.5 M MES (2-(N-morpholino) ethanesulphonic acid) adjusted to pH 6.3 with KOH

100 mM KCl

45 mM $MnCl_2$

10mM $CaCl_2$

3mM hexaminecobaltic chloride

2XTY.

16g bacto-tryptone

10g bacto-yeast extract

5g NaCl

adjusted to pH 7.4 and made up to 1L with DDW

Appendix 2

(Oligonucleotide sequences)

Probe Name	Forward and Reverse Primer Sequences	Product size	Annealing temperature
G6PD	F - attcatcatcatgggtgcatcg R - tgtttgcggatgtcagccactgt	168 b.p.	58 or 60^{0}C
β-Actin	F - tgctatccaggctgtgctat R - gatggagttgaaggtagttt	480 b.p.	58^{0}C
GluR-6 (1)	F - ccaggatagtgggaggcatttgg R - gccttcctcttgcagctgaagaattgc	469 b.p.	67^{0}C
GluR-6 (2)	F - ggcgcaccgttaaactcctgctctg R - gtagcaatgttctgtttctgttaattgtgttc	173 b.p.	65^{0}C

Table 2.A.1 *G6PD*, *β-Actin* and *GluR-6* specific forward (F) and reverse (R) primer sequences used for RT-PCR analysis.

Exon	Sequence of exon and position of primers	Product size	Annealing temperature
1	catcctcatt tctacccgaa cccaggagcc gaacgctaga tcggggaagt gggtgccgtg cgtgtgggca cagaaacacc **atgaagatta ttttcccgat tctaagtaat ccagtcttca ggcgcaccgt taaactcctg ctctgtttac tgtggattgg atattctcaa ggaaccacac atgtattaag atttg**gtaag attccccatc tctcttggtt gcctggtatc cgctcccagg cagccggatg taaacaggcg gttcgctctg cctggcggtg ggctccgtgg attctgatct	230 b.p.	65^0C
2	atataaaagt acagaaaact tctgatattt tctttatctt gtgaaaatta tgtttattct tgtaaaattt atgatttttc tctttctttt tgccag**gtgg tatttttgaa tatgtggaat ctggcccaat gggagctgag gaacttgcat tcagatttgc tgtgaacaca attaacagaa acagaacatt gctacccaat actaccctta cctatgatac ccagaagata aacctttatg atagttttga agcatccaag aaaggtaatt** gatagatttt taacatcttt gtttcctgga attcaaattt ctaggtattc ttaagagatt	280 b.p.	60^0C
3	tgttggcaca ctggcacctc tcctctcatt attgacagac ctttatctcc tcttcag**cct gtgatcagct gtctcttggg gtggctgcca tcttcgggcc ttcacacagc tcatcagcaa acgcagtgca gtccatctgc aatgctctgg gagttcccca catacagacc cgctggaagc accaggtgtc agacaacaaa gattccttct atgtcagtct ctacccagac ttctcttcac tcagccgtgc cattttagac ctggtgcagt ttttcaagtg gaaaaccgtc acggttgtgt atgatgacag cactg**gtaag aaaaatcagt atcttttgga gctatgcttt aaatatagat aatttctga agcccattcc aggttcttat tgtattctta	362 b.p.	65^0C
4	ttatacattc taatgagtgt tttctgattc tttgccctac tctatttttt atctctaata ttctttttt tttttccatt ttaattaaag **gtctcattcg tttgcaagag ctcatcaaag ctccatcaag gtataatctt cgactcaaaa ttcgtcagtt acctgctgat acaaaggatg caaaaccctt actaaaagaa atgaaaagag gcaaggagtt tcatgtaatc tttgattgta gccatgaaat ggcagcaggc attttaaaac** aggtaacctt taaattactt caattcttgt tttcttcata aacttgcact tacaatatga tcttctttta	294 b.p.	60^0C

5	gattctttat cacatcctac tatattttgt tctcacttga attactgaca tactgtcttt cctgaaatat gtaccttcag taatttttt ttttccttag **gcattagcta tgggaatgat gacagaatac tatcattata tctttaccac tctg**gtaagt acttcattaa aacaaaatgt tctgaatttt attattatga ctttttcaga tgaaaaatag gtttttagt aagaagtatt atgactgatt cctttgtagt tatattttaa	226 b.p.	60^0C
6*	tttcagtaat acatgcctgt gaagatactc tgtccataat aacaacacaa taacatttgt ctttcag**gac ctctttgctc ttgatgttga gccctaccga tacagtggtg ttaacatgac agggttcaga atattaaata cagaaaatac ccaagtctcc tccatcattg aaaagtggtc gatggaacga ttgcaggcac ctccgaaacc cgattcaggt ttgctggatg gatttatgac** ggtatgaata cccacttaaa gatcagtttg tgtgtttcta aatagtattg catacatatg aacttctggg tttatatgca tacatataaa cttcagttta	280 b.p.	60^0C
7	tcttccctct tcctccttgc aaaccatcta ccacaagttc tacaagttat attgactata aatttccctt tcccttgctt ttcag**actga tgctgctcta atgtatgatg ctgtgcatgt ggtgtctgtg gccgttcaac agtttcccca gatgacagtc agttccttgc agtgtaatcg acataaaccc tggcgcttcg ggacccgctt tatgagtcta attaaagagg** taagttagga gaagaacatc tgccttgtct cttttgttgt caagctgaat gaagtgagat tattgtggtt tttctagcaa gtgtcctttt actttatgtt	291 b.p.	65^0C
8	agcaatgtaa actgaaaagt aatgaatata agtttcctac ttttgtgaaa aaatgtttat tccatctttc ttcacttatt tattatcaat gtttttctat tcccatag**gc acattgggaa ggcctcacag gcagaataac tttcaacaaa accaatggct tgagaacaga ttttgatttg gatgtgatca gtctgaagga agaaggtcta gaaaaggtat** ttcagtgagc ttatttgctt taattactaa atgaaacata tctcaaagaa gacaaaatatt ctctgttgta gtcacatttc attgacattt	275 b.p.	60^0C

9	gtactctcta ccacgtgcct gatggacaat ttacaaagta catatgctat tttatagatt **ggaacgtggg atccagccag tggcctgaat atgacagaaa gtcaaaaggg aaagccagcg aacatcacag attccttatc caatcgttct ttgattgtta ccaccatttt** ggtaagtatt tgcttttcca ttattcttag ttaaatgtag atgaacacag aagtgcatag aaaaggacat	226 b.p.	60^0C
10	aagaaaagca ataattctga tgatgagttt catgattaac tgttacctct tttcttcttt ttcaatgttt tcag**gaagag ccttatgtcc tttttaagaa gtctgacaaa cctctctatg gtaatgatcg atttgaaggc tattgcattg atctcctcag agagttatct acaatccttg gctttacata tgaaattaga cttgtggaag atgggaaata tggagcccag gatgatgcca atggacaatg gaatggaatg gttcgtgaac taattgatca** tgtaagtccc ttccctcatg atttattagt ttgttatgtt gctacaaggt ttactctttt gttgatgtat	322 b.p.	60^0C
11*	ttaatttgat tactgatttt ttcttgtgac tgaaaatcaa ttttttctct ctttctttct ttctttttt ttttttttg tttgtttctg **tctacagaaa gctgaccttg cagttgctcc actggctatt acctatgttc gagagaaggt catcgacttt tccaagccct ttatgacact tggaataagt attttgtacc gcaagcccaa tggtacaaac ccaggcgtct tctccttcct gaatcctctc tcccctgata tctggatgta tattctgctg gcttacttgg gtgtcagttg tgtgctcttt gtcatagcca** ggtaacatgc tcacttttgt gatttttttg gcaattgtta cctcctttat ctaactaata	363 b.p.	60^0C
12	ttttgcagca aaaaatgctg gatagaattt cttcccactg caatttaaat gtattctttt ttctgtcaat taccacaggt **ttagtcctta tgagtggtat aatccacacc cttgcaaccc tgactcagac gtggtggaaa acaattttac cttgctaaat agtttctggt ttggagttgg agctctcatg cagcaaggta** tacgattcag cctgctattt cctttgggca ccatgtccca ctctttgctg ggggtgacag	237 b.p.	65^0C

13	aagtatctga ttgctgatca aattctctat attcgtttca cctttccccc actctctgtt **aggttctgag ctcatgccca aagcactgtc caccaggata gtgggaggca tttggtggtt tttcacactt atcatcattt cttcgtatac tgctaactta gccgcctttc tgacagtgga acgcatggaa tcccctattg actctgctga tgatttagct aaacaaacca agatagaata tggagcagta gaggatggtg caaccatgac ttttttcaag** gtaagttctg ctggttacct aaaatttaca aattaaaatg atagagcgca aacttctctc gattcacaaa	330 b.p.	65^0C
14	atgtagttca ttgtgtctaa gcattatagg agcacatgga aactaaagaa taactttctc gtgaccaact tatatttatt ttcttcagaa **atcaaaaatc tccacgtatg acaaaatgtg ggcctttatg agtagcagaa ggcagtcagt gctggtcaaa agtaatgaag aaggaatcca gcgagtcctc acctctgatt atgctttcct aatggagtca acaaccatcg agtttgttac ccagcggaac tgtaacctga cacagattgg cggccttata gactctaaag gttatggcgt tggcactccc atggg**gtaggt tatatgtcag ctctttgttc ttgttcatt aatctgagtt gctgtaagac aggggaaaaa	352 b.p.	65^0C
15	cattttgaaa aatctctgcc ctcctctcat cttgctaacc tttaattatg aaatttcagg ttccttgaaa tacttaacat aacctctcct ttcttag**gtt ctccatatcg agacaaaatt accatagcaa ttcttcagct gcaagaggaa ggcaaactgc atatgatgaa ggagaaatgg tggaggggca atggttgccc agaagaggag agcaaagagg ccagtgccct gggggttcag aatattggtg gcatcttcat tgttctggca gccggcttgg tgctttcagt ttttgtggca gtgggagaat ttttatacaa atccaaaaaa aacgctcaat tggaaaaggt** aaatgttact tgtttcagtt taaatttaaa acaatttttg ttgttacaat aaaacacaaa ccaaaagagt ttttatgtta ccaactaatg ataatgcata gaactattgc tctaaattgt	432 b.p.	60^0C
15b	caagatttcc agatctacat tgtttcaaga attagagatg tgtaaatata aattgactaa ttaaatataa tgaattttat taagagtttt actaatttaa atgcaattat acatgctaac agctcaaaga tttaaatttt cattttcag**g aatcttctat ttggttagtg ccaccatacc atccagacac tgtttagtaa tcttttgaaa cttactaaaa gaggttttta ataatg**gtta gtatccttgt gcctttcttt tttcttttg tagtctgtta tattaatgtg gagaaacatt tttattgttg ttttcatcct aaggaactga ccttatttaa ttctccttct	324 b.p.	60^0C

16	tgttgaggat gattttaata ttgatcttgg acagttacag tttatgtatc ttgtaatatt taatttttct gtgttcttct **gtagaggtcc ttctgtagtg ccatggtaga agaattgagg atgtccctga agtgccagcg tcggttaaaa cataagccac aggccccagt tattgtgaaa acagaagaag ttatcaacat gcacacattt aacgacagaa ggttgccagg taaagaaacc atggcataa**a gctgggaggc caaacaccca agcacaaact gtcgtctttt tccaaaacaat ttagccagaa tgtttcctgt ggaaatatgc	301 b.p.	60^0C

Table 2A.2 Sequence of exons (bold type) and flaking intronic regions of *GluR-6*. The positions of forward and reverse primers used to amplify each exon for sequence analysis are highlighted in grey. * The position of the second set of exon 6 primers is highlighted. *The position of nested primers used to amplify exon 11 are marked in a lighter shade of grey.

Probe Name	Forward and Reverse Primer Sequences	Nucleotide position	Exons encompassed
IGF2R SB 1	F - tagggatcccgcagtccgggcccggcg R – tagggatccggtgcagtcaacctcccct	1- 1628	1-11
IGF2R SB 2	F - taggaattccagcggatgagcgtcataaac R – taggaattcggtttctgcctcacagccag	1360 - 3239	10 - 22
IGF2R SB 3	F - taggaattctgtctgcggcacaatgcctg R – taggaattccttgtacaccagctgcaggac	3047 - 5095	22 - 34
IGF2R SB 4	F - taggaattcgcgtggcaaggccaacaag R – taggaattcacgagactccgttgtggaccag	4839 - 6216	34 - 40
IGF2R SB 5	F - taggaattctgggaggccacaagtcttcagt R – taggaattcgaagcatcatcgagtggaagtcttat	5982 - 9091	40 - 48
SP6	F - ttccaagaccccgagtcaatgcctaaaacc		

Table 2A.3 Paired oligonucleotide sequences used to amplify probes for Southern blot analysis of the *IGF2-R*.

Primer	Sequence
SMART II	AAGCAGTGGTAACAACGCAGAGTACGCGGG
5'-CDS	$(T)_{25}N_{-1}N$ [where N=A, C, G, or T ; N_{-1} =A, G or C]
3'-CDS	AAGCAGTGGTAACAACGCAGAGTAC$(T)_{30}N_{-1}N$

Table 2.A.4 Primer sequences used for first strand c-DNA synthesis for SMART RACE PCR.

Primer	Sequence
Long Universal primer	ctaatacgactcactatagggcaagcagtggtaacaacgcagagt
Short universal primer	ctaatacgactcactatagggc
Nested universal primer	aagcagtggtaacaacgcagagt
IGF2R exon 1	agcagtcgcgcgccgttagcctc
IGF2R exon 2	acatctgtggaagtgtggatattgtcc
IGF2R exon 2b	tacatgggaagctgttgataccaaaataat
IGF2R exon 3	tgagaagtgcaaccagatctctcctg
IGF2R exon 7	tgcctcttccctcacgtaactcagg
IGF2R exon 8	tctatggagacatcctgctgctcgc
IGF2R exon 9	tgctttgacttgagaggtatcgctctt
IGF2R exon 10	tgaaaccctgagctgcattcatcacc
MRP8-F	gtctcttgtcagctgtctttcagaag
MRP8-R	tatcaccagaatgaggaactcctgga
MRP8 nested-R	ctgcaccatcagtgttgatatccaac

Table 2A.5 Oligonucleotide sequences used for SMART RACE PCR analysis.

Appendix 3

(Characteristics of Genes on 6q)

Gene	Ensemble I.D.	Transcript size (b.p.)	Number of exons	Size of Genomic region (Kb)
POU3F2	ENSG00000132419	1329	1	1.329
FBXL4	ENSG00000112234	1353	6	43.44
Q9BXE6	ENSG00000164415	372	1	0.372
Novel	ENSG00000146273	771	1	0.771
Q9B552	ENSG00000146267	1512	6	68.49
NM017421	ENSG00000132423	1335	7	24.81
Q9POCO	ENSG00000132424	2422	11	23.85
Novel	ENSG00000152263	1381	8	38.99
Q9BRU1	ENSG00000123552	1387	8	33.17
CCNC	ENSG00000112237	2145	12	26.27
PRDM13	ENSG00000112238	2187	4	7.72
Q9BXA8	ENSG00000152034	1020	5	35.21
SIM I	ENSG00000112246	3995	11	274.8
L043738	ENSG00000112214	5748	39	496.44
Q9H5A2	ENSG00000112254	336	3	8.89
GluR-6	ENSG00000164418	2727	17	670.0

Table 3A.1 Transcript length, number of exons and size of genomic region covered by genes positioned within the CDR derived from acute leukemia patients with cytogenetic deletions of 6q. Genes are listed according to position in a centromeric to telomeric orientation.

Gene	Structure and function of gene
POU3F2	Also known as Brain Specific homeobox/POU domain protein 2 (BRN-2) or Nerve system specific octamer-binding transcription facter N-OCT 3. Member of 8 member POU domain protein family.
FBXL4	Putative protein fragment of unknown function with no other known family members.
Q9BXE6	Function unknown, no other known family members.
Novel	Oxidoreductase, member of 2 member oxidoreductase family.
Q9B552	Hypothetical 56 KDA protein fragment of unknown function with no Other known family members.
NM017421	Hexaprenyldihydroxybenzoate methyltrannsferase.
Q9POCO	Function unknown, no other known family members.
Novel	Ubiquitin carboxy terminal hydrolase 16 (Ubiquitin processing protease). Member of ubiquitin carboxy terminal hydrolase family.
Q9BRU1	Hypothetical 32.7 KDA protein of unknown function with no other family members.

CCNC	Cellular cyclin, binds to and activates cyclin dependent kinase 8 that phosphorylates a sub-unit of RNA polymerase II. Positive regulator of cell cycle transition.
PRDM13	PR-domain zink finger protein 13, no other known family members.
Q9BXA8	Melanin concentrating hormone receptor 2 (MCH2) G protein-coupled receptor, expressed predominantly in the brain and thought to be involved in the regulation of food intake and Energy balance.
SIM I	Homologue of (*Drosophila*) single minded 1. bhlh transcription factor, involved in embryogenesis.
L043738	RNA helicase (fragment), member of U5 small ribonuclear protein Family.
Q9H5A2	Novel protein of unknown function.
GluR-6	Glutamate receptor 6 sub-unit, also known as GRIK 2, member of ionotropic glutamate receptor family. Prominent role in neurotransmission.

Table 3A.2. Description of the genes contained within the CDR derived from FISH analysis of cases of acute leukemia with cytogenetic deletion of 6q.

Gene	Ensemble I.D.	Transcript size (b.p.)	Number of exons	Size of Genomic region (Kb)
HEY2	ENSG00000135547	2533	5	11.55
Novel	ENSG00000146375	2535	12	67.82
Q9NQES	ENSG00000111912	303	3	1.11
Q9NQE9	ENSG00000111911	2536	5	22.69
Q9NQF0	ENSG00000066651	2079	14	52.79
Novel	ENSG00000152872	320	4	66.54
Q9BXY4	ENSG00000146374	2390	5	78.39
Q9NTX6	ENSG00000118518	2146	4	21.49
Q9NTX5	ENSG00000093144	2123	6	53.70
043158	ENSG00000009718	6302	4	10.60
Q9UGC1	ENSG00000118509	2022	6	42.50
Novel	ENSG00000152884	915	5	13.14
Novel	ENSG00000164460	300	3	103.03
PTPRK	ENSG00000152894	2844	18	524.90
Q9UN81	ENSG00000164481	1014	1	1.014
LRE1	ENSG00000164481	3825	1	3.825
LAMA2	Not available	9534	67	400.0
Q96564	ENSG00000146376	3297	14	64.93
Q96JM7	ENSG00000146381	4101	22	
Novel	ENSG00000164483	590	4	30.86
Q96PX5	ENSG00000164484	1503	1	1.503
EPB41L2	ENSG00000079819	4290	19	117.16
AKAP7	ENSG00000118507	978	7	136.39
ARG1	ENSG00000118520	1447	8	
CRSP3	ENSG00000112282	5215	29	41.48
Q9NTTS	Not available	-	-	-

Novel	ENSG00000154269	1931	13	62.47
OR2A4	ENSG00000135570	930	1	0.930
095046	ENSG00000135570	2331	1	2.331
ENPP1	ENSG00000112264	3511	25	83.19
Novel	ENSG00000164486	327	1	0.327
Novel	ENSG00000146384	381	1	0.381
CTGF	ENSG00000118523	1047	5	1.91
Q9Y4U3	ENSG00000079931	2950	11	79.00
STX7	ENSG00000079950	1588	9	43.58
GPR102	ENSG00000146385	1029	1	1.029
Q96R18	ENSG00000135569	1035	1	1.035
014804	ENSG00000135569	1011	1	1.011
GPR58	ENSG00000146378	918	1	0.918
Q96RJ0	ENSG00000146399	1017	1	1.017
VNN1	ENSG00000112299	3106	7	32.4
VNN3	ENSG00000093134	1711	7	11.97
VNN2	ENSG00000112303	2004	7	14.03
N_M052831	ENSG00000146409	2367	14	29.18
RPS12	ENSG00000112306	501	6	3.00
Q9UN80	ENSG00000154272	3825	1	3.825
Q9UN81	ENSG00000145152	1014	1	1.014
Q9NTR7	ENSG00000112319	1842	17	82.18
Novel	ENSG00000153889	471	1	0.471
Q9BXE6	ENSG00000164473	372	1	0.372
TCF21	ENSG00000118526	1254	2	3.12
TBPL-1	ENSG00000028839	558	6	6.91
Q96MR8	ENSG00000146411	1329	4	27.7
SGK	ENSG00000118515	2354	12	5.6
Novel	ENSG00000153894	204	1	0.204
ALDH8A1	ENSG00000118514	2536	7	32.7
HBS1L	ENSG00000112339	2046*	17	88.37

L MYB	ENSG00000118513	3676*	16	37.86
Q9NXT1	ENSG00000135541	5267*	26	208.27
Novel	ENSG00000164478	372	1	0.372
Novel	ENSG00000164479	132	1	0.132
Q9BZ40	None available	1266	11	90.00
Q9BZ38	ENSG00000146410	1152	5	11.86
Q9NYF8	ENSG00000029363	5538*	13	31.27
MAP7	ENSG00000135525	2238*	17	204.55
MAP3K5	ENSG00000008177	4137	29	234.4
PEX7	ENSG00000112357	1448	10	91.34
Novel	ENSG00000146391	885	2	1.9
IL2ORA	ENSG00000016402	3485	7	45.00
Q96A41	ENSG00000164485	2754*	7	29.69
	ENSG00000146394	561	1	0.561
IFNGR1	ENSG00000027697	2096	7	21.93
Novel	ENSG00000146392	768	1	0.768
TNFAIP3	ENSG00000118503	4425	9	15.86
Q9H230	ENSG00000112378	1875	3	16.59
Novel	ENSG00000164422	327	4	42.43
Q9GZY1	ENSG00000118531	405	1	0.405
Novel	ENSG00000164423	344	4	0.344
Q9ULH6	ENSG00000112379	5421	24	74.96
C6orf34	ENSG00000051620	1197	4	9.16
Q9P2J0	ENSG00000135540	4023	3	9.47
Novel	ENSG00000151992	687	4	124.92
Q9Y430	ENSG00000024862	1245	6	19.55
NM_016557	ENSG00000118519	1858	1	1.858
REPS1	ENSG00000135597	2435	19	43.05
Q9P1F3	ENSG00000146386	818	3	14.6
Q9UB19	ENSG00000112403	5614	4	45.69
Novel	ENSG00000164440	2055	10	46.37

Q14287	ENSG00000151997	471	1	0.471
Q9C063	ENSG00000151998	660	9	51.29
Novel	ENSG00000164443	153	1	0.153

Table 3A.3 Transcript length, number of exons and size of genomic region covered by genes positioned within the region of 6q containing a cluster of leukaemia related translocation break points. Genes are listed according to position in a centromeric to telomeric orientation. * more than one splice form of this gene, size and exon number presented are for the largest transcript.

Gene	Structure and function of gene
HEY2	Hairy/enhancer of split related (*Drosophila*) with YRPW motif 2. BHLH protein Expressed during embryogenesis
Novel	Unknown function
Q9NQES	Novel protein similar to *Drosophila* L82.
Q9NQE9	Novel protein similar to protein kinase C inhibitors.
Q9NQF0	Novel protein similar to S PO.
Novel	Function unknown
Q9BXY4	Thrombospodin type 1, member of 3 member family.

Q9NTX6	Novel C3HC4 type zinc finger (ring finger) protein.
Q9NTX5	Uncharacterised hypothalamus protein.
043158	Function unknown.
Q9UGC1	Function unknown.
Novel	Function unknown
Novel	Function unknown
PTPRK	Protein-tyrosine phosphatase, receptor type kappa. Involved in the Regulation of cell contact and adhesion, growth control, tumour Invasion and metastasis.
Q9UN81	Function unknown
LRE1	Contains a reverse transcriptase domain, member of a 300 member LINE 1 reverse transcriptase family.
LAMA 2	Laminin alpha 2, also known as merosin. Mutations are the cause of congenital merosin-deficient muscular dystrophy. Thought to mediate the attachment, migration and organization of cells into tissues during embryonic development.
Q96564	RhoGAP containing protein, function unknown.
Q96JM7	Function unknown.

Novel	Function unknown
Q96PX5	Function unknown
EPB41L2	Erythroid membrane protein band 4.1-like 2, also known as generally expressed protein 4.1 (4.1 G). Major structural element of the erythrocyte membrane skeleton which stabilizes spectrin-actin interaction. Also associates with the nuclear mitotic apparatus. Widely expressed.
AKAP7	A-kinase anchor protein 7. Targets the cAMP-dependent protein kinase (pka) to the plasma membrane.
ARG1	Arginase 1 (liver type arginase), essential component of the Urea cycle.
CRISP3	Cofactor required for Sp1 transcriptional activation subunit 3, vitamin D3 receptor interacting protein.
Q9NTTS	Phosphodiesterase 1 nucleotide pyrophosphotase 3 (PDNP3).
Novel	Ectonucleotide pyrophosphatase / phosphodiesterase 1(ENPP1), plasma membrane glycoprotein PC1.
OR2A4	Olfactory receptor like protein
095046	Meningioma expressed antigen 6/11, transcription factor.
ENPP1	Ectonucleotide pyrophosphatase / phosphodiesterase 1, may have a role In regulation of n-glycosylation.
Novel	Function unknown

Novel	Function unknown
CTGF	Connective tissue growth factor precursor, also known as hypotrophic Chondrocyte-specific protein 24 and insulin-like growth factor-binding Protein (IGFBP8). Promotes proliferation and differentiation of Chondrocytes.
Q9Y4U3	Monooxygenase X.
STX7	Syntaxin 7, mediator of endocytic trafficking.
GPR102	Trace amine receptor.
Q96R18	Trace amine receptor.
014804	Trace amine receptor.
GPR58	Trace amine receptor.
Q96RJ0	Trace amine receptor.
VNN1	Vascular non-inflammatory molecule 1 (VANIN 1), involved in the recycling of pantathenic acid. Expressed in spleen, thymus, peripheral blood lymphocytes and small intestine.
VNN3	Vascular non-inflammatory molecule 3 (VANIN 3), thought to be a hydrolase involved in the control of lymphocyte migration into tissues.
VNN2	Vascular non-inflammatory molecule 2 (VANIN 2), involved in the homing of bone marrow cells to the thymus. May regulate beta-2

	integrin-mediated cell adhesion, migration and motility of neutrophils.
NM_052831	Function unknown
RPS12	40S Ribosomal protein S12.
Q9UN80	Line 1 reverse transcriptase, member of 300 member family.
Q9UN81	Function unknown.
Q9NTR7	Eyes absent homologue (*Drosophila*) 4 (EYA 4), member of four Member family. Mutations are cause of late onset deafness.
Novel	Line 1 reverse transcriptase, member of 300 member family.
Q9BXE6	Function unknown
TCF21	Transcription factor 21, also known as Podocyte expressed 1 (POD-1), Epicardin or Capsulin. 2 members in family.
TBPL-1	TATA box binding protein like protein 1, also known as TATA box binding protein-related factor 2 or Stud protein. Ubiquitously expressed DNA binding protein.
Q96MR8	Similar to glucose transporter type 2, member of 2 member solute carrier family.
SGK	Serum /glucocorticoid-regulated kinase (serine/threonine-protein Kinase), member of 23 member family.
Novel	Function unknown, has a bipartite nuclear localization signal.

ALDH8A1	Aldehyde dehydrogenase family member 8 A1 (13 members in family)
HBS1L	HBS1 (*S.Cerevisiae*)-like protein, member of 17 member Elongation factor 1 alpha family. Contains GTP binding elongation factor and ATP /GTP- binding site motif A domains.
L-MYB	MYB proto-oncogene protein (C-MYB), expressed at high levels in early lymphocyte development and reported to be deregulated, as a result of translocation or mutation, in leukemia. Member of, 3 member family.
Q9NXT1	Function unknown, contains SH3, RecA bacterial recombination protein and G-protein beta WD-40 repeat domains.
Novel	Function unknown.
Novel	Glyceraldehyde 3 phosphate dehydrogenase, member of, 11 member family.
Novel	Glyceraldehyde 3 phosphate dehydrogenase, member of, 11 member family.
Q9BZ40	High affinity cAMP-specific 3',5'-cyclic phosphodiesterase (Roliprim Insensitive phosphodiesterase type 7)
Q9BZ38	Novel protein, function unknown.
Q9NYF8	BCL-2-associated transcription factor.
MAP7	Microtubule associated protein 7, predominantly expressed in epithelial

	cells.
MAP3K5	Mitogen activated protein kinase, kinase, kinase 5 (MEKK5) also Known as Apoptosis signal regulator kinase 1 (ASK). Member of 2 member serine/threonine protein kinase family. Overexpression induces apoptosis.
PEX 7	Peroxisomal targeting signal 2 receptor (PTS2 receptor) also known as peroxin. Plays an essential role in peroxisomal protein import. Defects in PEX 7 are the cause of rhizomelic chondrodysplasia punctata type 1.
Novel	UDP glucuronic acid/UDP N acetylgalactosamine transporter (UDP GLCA/UDP GALNAC), member of 3 member family.
IL2ORA	Class II cytokine receptor 2CYTOR 7 also known as interleukin 20 receptor alpha.
Q96A41	Soluble cytokine class II receptor also known as interleukin 22-binding protein.
PRO1828	Function unknown
IFNGR1	Interferon-Gamma receptor alpha chain precursor (IFNGR1), member Of class II cytokine receptor family.
Novel	Protein kinase C binding protein Rack 17 has bHLH dimerisation Domain.
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3, also known as Zink Finger protein A20. Inhibitor of tnf-induced gene expression and apoptosis. Has a role in the function of the lymphoid system.

Q9H230	P53-induced protein PIGPC1.
Novel	Function unknown.
Q9GZY1	UC28 protein.
Novel	Function unknown
Q9ULH6	KIAA1244 protein (fragment), function unknown.
C6orf34	Putative heme binding protein.
Q9P2J0	KIAA1357 protein (fragment), function unknown.
Novel	Function unknown.
Q9Y430	Hypothetical protein fragment, function unknown.
NM_016557	CC Chemokine receptor type II (C-C CKR-II) also known as chemokine receptor-like 1 (CCRL1). Member of 15 member family.
REPS1	Function unknown
Q9P1F3	Hypothetical protein.
Q9UB19	Homologue of *Drosophila* Head case.
Novel	Specific response 5 (member of 5 gene family)

Q14287	Member of 300 member LINE 1 reverse transcriptase family.
Q9C063	LYST-interacting protein (LIP5)
Novel	Member of 3 member ATP synthase B chain, family.

Table 3A.4 Function of genes positioned within the region of 6q containing a cluster of leukaemia related translocation breakpoints.

Appendix 4

The nucleotide position of exons of the IGF2-R and restriction enzyme sites on sequenced clones.

Exon	Probe	Position on IGF2R cDNA	Position on clone RP1-249F5	Position on clone RP1-288H12	Position BamH1 sites and fragment sizes		Position EcoR1 sites and fragment sizes	
					49981			
					44406	5575	44926	
1	1	1-209	43988-43780		31221	13185	36991	7935
					26591	4630	32286	4705
							28946	3340
2	1	298-437	21903-21764		16911	9680	17281	11665
					14636	2275	15041	2240
					13946	690	10050	4991
					8065	5881	9790	260
					4860	3205		
3	1	438-562	4077-3953		3135	1725	4035	5755
							3005	1030
							2630	375
4	1	563-661	2400-2340					
							2860	5490
							3785	925
					10236	13371		
							5421	1636
							6391	970
							7111	720

						12836	5725
						14101	1265
5	1	662-794	11586-11718				
6	1	795-924	14199-14328				
7	1	925-1030	16564-16669	19066	8830	17371	3270
8	1	1031-1193	19565-19727				
9	1	1194-1359	19956-20121	27261	8195	24706	7335
10	1/2	1360-1463	21433-21536	28036	775		
11	1/2	1464-1628	27574-27738				
12	2	1629-1769	30162-30302				
13	2	1770-1913	31528-31671	33316	5280	33161	8455
14	2	1914-2051	32759-32896				
15	2	2052-2199	33512-33659				
16	2	2200-2377	34173-34350				
17	2	2378-2493	34806-34921			35071	1910
18	2	2494-2662	35389-35557	37636	4320	35831	760
				39916	2280	36551	720
				42181	2265	36651	100
19	2	2663-2842	37487-37666	45056	2875	37121	470
20	2	2843-2944	43438-43539			43186	6065
21	2	2945-3046	45037-45138				
22	2/3	3047-3239	45920-46112	47661	2605		
23	3	3240-3410	47561-47731				
24	3	3411-3554	48517-48660				
25	3	3555-3730	48767-48942				
26	3	3731-3818	49546-49633				
27	3	3819-4034	50429-50644				
28	3	4035-4165	51415-51545				
29	3	4166-4263	51818-51915			56507	13321
30	3	4264-4400	55263-55399				
31	3	4401-4591	56882-57072				
32	3	4592-4718	58924-59050				

33	3	4719-4838	59779-59898				
34	3/4	4839-5095	60227-60483				
35	4	5096-5314	60771-60989	61741	14080		
36	4	5315-5464	62861-63010	61946	205	64716	8209
37	4	5465-5626	65215-65376				
38	4	5627-5834	66594-66801				
39	4	5835-5981	67143-67289				
						67393	2677
						68573	1180
40	4/5	5982-6216	70964-71198			68771	198
41	5	6217-6353	72009-72145			72806	4035
42	5	6354-6468	75048-75161			73676	870
43	5	6469-6615	76121-76267			75235	1559
44	5	6616-6803	76930-77117	79451	17505	76466	1231
45	5	6804-6990	83453-83639	83561	4110	82981	6515
				84991	1430	84711	1730
46	5	6991-7143	89533-89685	85206	215		
47	5	7144-7213	90760-90829				
48	5	7214-9091	91688-92695	101475	16268		
						92886	8175

Table 4.A.1 Position of exons of the *IGF2-R* gene with respect to the 5 probes used for Southern blot analysis of patient 22, the DNA sequence of PACs RCPI1-249F5 and RCPI1-288H12, and predicted BamH1 and EcoR1 restriction enzyme sites and fragment sizes. Fragments highlighted in red include one or more *IGF2-R* exons. Green fragments cover only intronic sequence and Purple fragments hybidise to part of an exon.

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